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(54) Title: **MEDICAMENTS**

**Nucleic acid sequence for rabbit HM74A**

MNQHRPQSHFLEIDKKNCCVFRDDFIANVLPPVLGLEFVFGLLGNGLALWIFCFHLKSWKSSR  
IFLFNLA VADFLLIICLPFLTDNYMRKWD WRFGDIPCRMLFMLAMNRQGSIFLTVVAVDYR  
FRV VHPHHALNKISNRTAA AISCLLWGV TIGLTVHLLRKRMLTQNGPANLCCSFSICNTFRWH  
DAMFLLEFFLPLAILFC SVRIVWSLRQRQMDRHVKIKRAINFIMVVA VVFICFLPSVAVRMRI  
FWLLRTAGTQDCD VYRSVDLAFFITLSFTYMNSMLDPLVYYFSSPSFPNFFSALINRCLRRSPA  
GEPENNRSTSVELTGDPSTARSAPDALVAEPNGPRSPSYLVPNPR

(57) Abstract: A method of treating HM74A and/or HM74 mediated disease in an individual which comprises administering to the individual a therapeutic amount of a modulator of HM74A and/or HM74 activity.

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## **MEDICAMENTS**

### **Field of the Invention**

5           This invention relates to the identification of the closely related orphan G-protein coupled receptors HM74A and HM74 as high and low affinity receptors, respectively, for the ligand nicotinic acid and their use in screening methods, and rational drug design, to identify modulators of these receptors, to the modulators so identified and their use in therapy. The present invention also relates to a method of  
10   treatment of diseases mediated by HM74 and HM74A comprising the administration of a therapeutically effective amount of a modulator of the receptors HM74 and HM74A.

### **Background of the Invention**

15           G-protein coupled receptors (GPCRs), otherwise known as 7TM receptors are a super-family of membrane receptors that mediate a wide variety of biological functions. Upon binding of extracellular ligands, GPCRs interact with a specific subset of heterotrimeric G proteins that can, in their activated forms, inhibit or  
20   activate various effector enzymes and/or ion channels. All GPCRs are predicted to share a common molecular architecture consisting of seven transmembrane helices linked by alternating intracellular and extracellular loops. The extracellular receptor surface has been shown to be involved in ligand binding whereas the intracellular portions are involved in G protein recognition and activation. Different G-protein  $\alpha$ -  
25   subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. G-protein coupled receptors are found at numerous sites within a mammalian host. Over the past 15 years many therapeutic agents targetting 7TM receptors have been successfully introduced onto the market, thereby establishing their value as therapeutic targets.

30           Activation of receptors coupled to the  $G_i$  family of G proteins leads to inhibition of adenylate cyclase and lowering of intracellular cAMP levels. In adipose tissue a reduction of cAMP levels results in an inhibition of hormone-sensitive lipase (HSL) activity, an enzyme which regulates the process of lipolysis (i.e. the hydrolysis of triglycerides (TG) to glycerol and non-esterified fatty acids (NEFA)).

Inhibition of adipocyte lipolysis resulting in a reduction of plasma NEFA levels is thought to reduce hepatic triglyceride synthesis resulting in a decreased output of TG-rich lipoproteins (VLDL). A reduction in VLDL synthesis would then produce a functional inhibition of cholesterol ester transfer protein (CETP) activity resulting in an elevation in high-density lipoprotein (HDL) levels. This alteration in lipoprotein levels, decreased TG and increased HDL, would be suitable for the treatment of dyslipidemic patients and should result in a decreased risk of cardiovascular disease. Furthermore, inhibition of adipocyte lipolysis via the activation of an, as yet, undefined Gi-coupled receptor is thought to be an important mechanism of action of nicotinic acid (Niacin). Nicotinic acid has been used clinically for over 40 years in patients with various forms of hyperlipoproteinaemia. Nicotinic acid produces a very desirable alteration in lipoprotein profiles; reducing levels of VLDL, LDL and Lp(a) whilst increasing HDL. Nicotinic acid has also been demonstrated to have disease modifying benefits, reducing the progression and increasing the regression of atherosclerotic lesions and reducing the number of cardiovascular events in several trials.

### **Summary of the Invention**

The present invention is based on the finding that the G-protein coupled receptors HM74A and HM74 are able to act as receptors for nicotinic acid and that cells transfected to express HM74A and/or HM74 gain the ability to elicit G<sub>i</sub> G protein mediated responses following exposure to nicotinic acid. Identification of a ligand for HM74A and/or HM74 therefore facilitates the development of screening methods for identifying modulators of the receptor.

Accordingly the invention further provides a method for identifying agents which modulate the activity of the HM74A and/or HM74 receptor, which comprises determining whether the test agent interacts with HM74A and/or HM74. The method may comprise the use of the HM74A and/or HM74 receptor in combination with a ligand therefore, e.g. nicotinic acid.

The invention further comprises the use of agents identified using the method of the invention in the treatment of diseases mediated by HM74 and/or HM74A and their

use in the manufacture of a medicament for the treatment of HM74 and/or HM74A mediated diseases.

Accordingly, the invention further provides a method of treatment of diseases or conditions mediated by HM74A and/or HM74 in an individual which comprises the administration of a therapeutically effective amount of an HM74A and/or HM74 receptor modulator. The present invention excludes by specific proviso the use of nicotinic acid in the method of the invention. The invention also provides the use of a modulator of HM74A and/or HM74 in the manufacture of a medicament for the treatment of diseases or conditions mediated by HM74A or HM74 with the proviso the modulator is not nicotinic acid.

#### **Brief Description of the Figures**

Fig. 1. Co-expression of HM74, but not of other orphan receptors, with  $G_{o1}\alpha$  in HEK293T cells results in nicotinic acid-mediated stimulation of [ $^{35}$ S]GTP $\gamma$ S binding

Fig. 2. Taqman mRNA analysis demonstrate distribution of HM74/HM74A in adipose, omentum and spleen.

Fig. 3. Nicotinic acid stimulates [ $^{35}$ S]GTP $\gamma$ S binding in membranes from HEK293T cells co-expressing HM74 and  $G_{o1}\alpha$  in a concentration-dependent manner

Fig. 4. Nicotinic acid, but not its metabolite nicotinuric acid, can stimulate [ $^{35}$ S]GTP $\gamma$ S binding in membranes from HEK293T cells co-expressing HM74 and  $G_{o1}\alpha$

Fig. 5. Nicotinic acid-mediated stimulation of [ $^{35}$ S]GTP $\gamma$ S binding in membranes from HM74-expressing HEK293T cells is sensitive to pertussis toxin pretreatment

Fig. 6. HM74 couples equally well to the  $G_i$ -like G proteins  $G_{i1}\alpha$ ,  $G_{i2}\alpha$ ,  $G_{i3}\alpha$  and  $G_{o1}\alpha$  following co-expression in HEK293T cells

Fig. 7. Nucleotide sequence of murine HM74A

Fig. 8. Amino acid sequence of murine HM74A

Fig. 9. Alignment of murine HM74A and human HM74 protein sequences

Fig. 10. cDNA sequence of rat HM74A

5 Fig. 11. Amino acid sequence of rat HM74A

Fig. 12. Amino sequence alignment of murine and rat HM74A

Fig. 13. Alignment of rat HM74A and human HM74 amino acid sequences

Fig. 14. Alignment of human HM74 and HM74A amino acid sequences

10 Fig. 15. Amino acid sequence alignment of human HM74A and murine  
HM74A

Fig. 16. Amino acid sequence alignment of human HM74A and rat HM74A

Fig. 17. Rat and human HM74A elicit nicotinic acid responses with similar  
potency following expression in HEK293T cells together with  $G_{o1}\alpha$

15 Fig. 18 Rat (A) and human (B) HM74A display similar pharmacological  
profiles using nicotinic acid analogues following expression in HEK293T cells  
together with  $G_{o1}\alpha$

Fig. 19. Nucleotide (cDNA) sequence of monkey HM74A

Fig. 20. Amino acid sequence of monkey HM74A

Fig. 21. Nucleotide sequence of rabbit HM74A

20 Fig. 22. Amino acid sequence of rabbit HM74A

### **Detailed Description of the Invention**

25 Throughout the present specification and the accompanying claims the words  
"comprise" and "include" and variations such as "comprises", "comprising",  
"includes" and "including" are to be interpreted inclusively. That is, these words are  
intended to convey the possible inclusion of other elements or integers not  
specifically recited, where the context allows.

30 Prior to this invention, the utility of the HM74A and HM74 receptors was  
unknown. The discovery that nicotinic acid can act as a ligand indicates that diseases  
or conditions mediated by HM74A and/or HM74 include dyslipidemia including  
associated diabetic dyslipidemia and mixed dyslipidemia, heart failure,

hypercholesteremia, cardiovascular disease including atherosclerosis, arteriosclerosis, and hypertriglyceridemia, type II diabetes mellitus, type I diabetes, insulin resistance, hyperlipidemia, anorexia nervosa, obesity. It is also believed that the HM74 and HM74A receptors are involved in inflammation. Inflammation  
5 represents a group of vascular, cellular and neurological responses to trauma. Inflammation can be characterised as the movement of inflammatory cells such as monocytes, neutrophils and granulocytes into the tissues. This is usually associated with reduced endothelial barrier function and oedema into the tissues. Inflammation with regards to disease typically is referred to as chronic inflammation and can last  
10 up to a lifetime. Such chronic inflammation may manifest itself through disease symptoms. The aim of anti-inflammatory therapy is therefore to reduce this chronic inflammation and allow for the physiological process of healing and tissue repair to progress. Examples of inflammatory diseases or conditions include those of the joint, particularly arthritis (e.g. rheumatoid arthritis, osteoarthritis, prosthetic joint  
15 failure), or the gastrointestinal tract (e.g. ulcerative colitis, Crohn's disease, and other inflammatory bowel and gastrointestinal diseases, gastritis and mucosal inflammation resulting from infection, the enteropathy provoked by non-steroidal anti-inflammatory drugs), of the lung (e.g. adult respiratory distress syndrome, asthma, cystic fibrosis, or chronic obstructive pulmonary disease), of the heart (e.g.  
20 myocarditis), of nervous tissue (e.g. multiple sclerosis), of the pancreas, (e.g. inflammation associated with diabetes melitus and complications thereof, of the kidney (e.g. glomerulonephritis), of the skin (e.g. dermatitis, psoriasis, eczema, urticaria, burn injury), of the eye (e.g. glaucoma) as well as of transplanted organs (e.g. rejection) and multi-organ diseases (e.g. systemic lupus erythematosus, sepsis)  
25 and inflammatory sequelae of viral or bacterial infections and inflammatory conditions associated with atherosclerosis and following hypoxic or ischaemic insults (with or without reperfusion), for example in the brain or in ischaemic heart disease.

By the term HM74 and/or HM74A, mediated diseases it is meant those diseases where the modulation of HM74 and/or HM74A, by ligands other than nicotinic acid, results in a beneficial modification of the disease state.

5

HM74A and HM74 may therefore be used as screening targets for the identification and development of novel pharmaceutical agents for use in the methods of the invention. A modulator of HM74A and/or HM74 may be identified by contacting a test agent with a cell expressing on the surface the receptor HM74 and/or HM74A, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of an agent to said receptor, with an agent to be screened under conditions to permit binding to the receptor; and determining whether the agent binds to, and activates, or inhibits, the receptor, by detecting the presence or absence of a signal generated from the interaction of the compound with the receptor and thereby determining whether the test agent modulates HM74A and/or HM74 activity. This may be carried out in the presence of a labelled or unlabelled ligand, e.g. nicotinic acid.

For example a method for identification of an agent that modulates HM74A and/or HM74 activity comprises:

- (i) contacting a test agent with a cell, such as an adipocyte, which expresses HM74A and/or HM74 or a variant thereof which is capable of coupling to a G-protein; and
  - (ii) monitoring for HM74A and/or HM74 activity in the presence of a G-protein;
- thereby determining whether the test agent modulates HM74A and/or HM74 activity.

The test agent may be contacted in step (i) with cells that express HM74A and/or HM74 or a variant thereof. Alternatively, the test agent may be contacted in step (i) with membrane obtained from such cells.

A modulator of HM74 and/or HM74A may be identified by determining the inhibition of binding of a ligand to cells which have the receptor on the surface thereof, or to cell membranes containing the receptor, in the presence of a candidate compound, under conditions to permit binding to the receptor, and determining the amount of ligand bound to the receptor, such that a compound capable of causing reduction of binding of a ligand is an agonist or antagonist, in which method the ligand is nicotinic acid.

The invention also provides:

- 10       - a test kit suitable for identification of an agent that modulates HM74A and/or HM74 activity, which kit comprises:
  - (a)     HM74A and/or HM74 or a variant thereof which is capable of coupling to a G-protein; and
  - (b)     means for monitoring HM74A and/or HM74 activity.
- 15       - a method for identification of an agent that inhibits lipolysis, which method comprises contacting adipocytes *in vitro* with a test agent which modulates HM74A and/or HM74 activity and which has been identified by the method of the invention and monitoring lipolysis, thereby determining whether the test substance is an inhibitor of lipolysis;
- 20       - an activator of HM74A and/or HM74 activity or an inhibitor of lipolysis identified by a method of the invention, their use in therapy and pharmaceutical compositions comprising them.
- 25       - an activator of HM74A and/or HM74 activity or an inhibitor of lipolysis identified by a method of the invention or a polynucleotide which encodes HM74A and/or HM74 or a variant polypeptide, for use in a method of treatment of the human or animal body by therapy; and
- 30       - use of such an activator, inhibitor or polynucleotide in the manufacture of a medicament for the treatment of diseases or conditions



modulated by HM74 and/or HM74A, for example dyslipidaemia and conditions associated with dyslipidaemia, coronary heart disease, atherosclerosis, thrombosis or obesity, angina, chronic renal failure, peripheral vascular disease, stroke, type II diabetes metabolic syndrome (syndrome X) or inflammation.

The present invention relates to the use of human G-protein coupled receptors, HM74 and HM74A, and variants thereof. HM74 has been cloned previously (Nomura, H., Nielsen, B. W. and Matsushima, K. *Int. Immunol.* 5: 1239-1249, (1993)). HM74 receptor has the GenBank Accession number D10923. The variant of HM74 known as HM74A is also a preferred variant. This is described below and also in WO9856820. The terms HM74A and HM74 as used herein incorporate variants of HM74 and HM74A. HM74A and HM74 receptors for use in the screening methods of the invention include all species orthologues, eg, may be rodent, mouse, rabbit, monkey or human. Rabbit and monkey nucleic acid and amino acid sequences are depicted in Figs. 19 - 22. Human is especially preferred. The term "variant" refers to a polypeptide which has the same essential character or basic biological functionality as HM74A and/or HM74. The essential characters of HM74A and HM74 can be defined as that of G-protein coupled receptors. HM74A and HM74 couple to  $G_i$  G-protein. Thus, the term "variant" refers in particular to a polypeptide which activates  $G_i$ .

To determine whether a candidate variant has the same function as HM74A and/or HM74, the ability of the variant to activate  $G_i$ -protein can be determined. The effect of the candidate variant on  $G_i$  activation can be monitored. This can be carried out, for example, by contacting cells expressing the candidate variant with a ligand which activates  $G_i$ -protein when contacted with cells that express HM74A and/or HM74, and measuring a  $G_i$ -coupled readout. A control experiment is typically also carried out in which cells of the same type as those expressing the candidate variant, but expressing HM74A and/or HM74 instead, are contacted with the ligand and a corresponding  $G_i$ -coupled readout is measured. The effect attained by the candidate variant can then be directly compared with that attained by HM74A and/or HM74.

Alternatively, a variant polypeptide is one which binds to the same ligand as HM74A and/or HM74. That can be determined directly by contacting a candidate variant with a radiolabelled ligand that binds to HM74A and/or HM74 and monitoring binding of the ligand to the variant. Typically, the radiolabelled ligand can be incubated with cell membranes containing the candidate variant. The membranes can then be separated from non-bound ligand and dissolved in scintillation fluid to allow the radioactivity of the membranes to be determined by scintillation counting. Non-specific binding of the candidate variant may also be determined by repeating the experiment in the presence of a saturating concentration of non-radioactive ligand. Preferably a binding curve is constructed by repeating the experiment with various concentrations of the candidate variant. The ability to bind a ligand of HM74A and/or HM74 may also be determined indirectly as described below.

Typically, polypeptides with more than about 65% identity, preferably at least 80% or at least 90% and particularly preferably at least 95%, at least 97% or at least 99% identity, with the amino acid sequence of HM74 as published (Nomura *et al* *Int. Immuno* 5 1239-1249, 1993) or HM74A as described in WO9856820 or more preferably over a region of at least 20, preferably at least 30, at least 40, at least 60 or at least 100 contiguous amino acids or over the full length of the amino acid sequences are considered as HM74A and HM74 variants. The UWGCG Package provides the BESTFIT program which can be used to calculate identity (for example used on its default settings) (Devereau *et al* (1984) *Nucleic Acid Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate identity or line up sequences (typically on their default settings), for example as described in Altschul S.F. (1993) *J. Mol. Evol.* **36**: 290-300; Altschul, S.F. *et al* (1990) *J. Mol. Biol.* **215**: 403-10. Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

Variant polypeptides therefore include naturally occurring allelic variants. An allelic variant will generally be of human or non-human mammal origin, such as bovine or porcine origin. Alternatively, a variant polypeptide can be a non-naturally

occurring sequence. A non-naturally occurring variant may thus be a modified version of HM74A and/or HM74.

The amino acid sequence of HM74A and/or HM74 may be modified by  
 5 deletion and/or substitution and/or addition of single amino acids or groups of amino acids as long as the modified polypeptide retains the capability to function as a G-protein coupled receptor. Such amino acid changes may occur in one, two or more of the intracellular domains of HM74A and/or HM74 and/or one, two or more of the extracellular domains of HM74A and/or HM74 and/or one, two or more of the  
 10 transmembrane domains of HM74A and/or HM74.

Amino acid substitutions may thus be made, for example from 1, 2, 3, 4 or 5 to 10, 20 or 30 substitutions. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second  
 15 column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
		H F W Y
AROMATIC		

20

A variant polypeptide may be a shorter polypeptide. For example, a polypeptide of at least 20 amino acids or up to 50, 60, 70, 80, 100 or 150 amino acids in length may constitute a variant polypeptide as long as it demonstrates the functionality of HM74A and/or HM74. A variant polypeptide may therefore lack  
 25 one, two or more intracellular domains and/or one, two or more extracellular domains and/or one, two or more transmembrane domains. A variant polypeptide

may thus be a fragment of the full length polypeptide. A shortened polypeptide may comprise a ligand-binding region (N-terminal extracellular domain) and/or an effector binding region (C-terminal intracellular domain). Such fragments can be used to construct chimeric receptors preferably with another 7-transmembrane G-coupled receptor.

Variant polypeptides include polypeptides that are chemically modified, e.g. post-translationally modified. For example, such variant polypeptides may be glycosylated or comprise modified amino acid residues. They may also be modified by the addition of histidine residues, for example 6 or 8 His residues, or an epitope tag, for example a T7, HA, myc or flag tag, to assist their purification or detection. They may be modified by the addition of a signal sequence to promote insertion into the cell membrane.

The invention also utilises nucleotide sequences that encode HM74A and/or HM74 or variants thereof as well as nucleotide sequences which are complementary thereto. The nucleotide sequence may be RNA or DNA including genomic DNA, synthetic DNA or cDNA. Preferably the nucleotide sequence is a DNA sequence and most preferably, a cDNA sequence. Such nucleotides can be isolated from human cells or synthesised according to methods well known in the art, as described by way of example in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbour Laboratory Press, 1989. Typically a useful polynucleotide comprises a contiguous sequence of nucleotides which is capable of hybridising under selective conditions to the coding sequence or the complement of the coding sequences of HM74A and/or HM74.

A polynucleotide can hybridize to the coding sequence or the complement of the coding sequences of HM74A and/or HM74 at a level significantly above background. Background hybridisation may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between a polynucleotide and the coding sequence or complement of the coding sequence of HM74A and/or HM74 is typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of HM74A and/or HM74. The intensity of interaction may be measured,

for example, by radiolabelling the probe, e.g. with  $^{32}\text{P}$ . Selective hybridisation may typically be achieved using conditions of low stringency (0.3M sodium chloride and 0.03M sodium citrate at about 40°C), medium stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 50°C) or high stringency (for example,  
5 0.03M sodium chloride and 0.003M sodium citrate at about 60°C).

The coding sequences of HM74A and/or HM74 may be modified by one or more nucleotide substitutions, for example from 1, 2, 3, 4 or 5 to 10, 25, 50 or 100 substitutions. The polynucleotides of HM74A and/or HM74 may alternatively or  
10 additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends. The modified polynucleotides generally encode polypeptides which have G-protein coupled receptor activity or inhibit the activity of HM74A and/or HM74. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the  
15 modified sequences are translated, for example as shown in the Table above.

A nucleotide sequence which is capable of selectively hybridising to the complement of the DNA coding sequences of HM74 and HM74A will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%  
20 or at least 99% sequence identity to the coding sequence of HM74 and HM74A over a region of at least 20, preferably at least 30, for instance at least 40, at least 60, more preferably at least 100 contiguous nucleotides or most preferably over the full length.. Methods of measuring nucleic acid and protein homology are well known in the art. For example the UWGCG Package provides the BESTFIT program which  
25 can be used to calculate homology (Devereux *et al* 1984). Similarly the PILEUP and BLAST algorithms can be used to line up sequences (for example are described in Altschul 1993, and Altschul *et al* 1990). Many different settings are possible for such programs. In accordance with the invention, the default settings may be used.

30 Any combination of the above mentioned degrees of sequence identity and minimum sizes may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher sequence identity over longer lengths) being preferred. Thus, for example a polynucleotide which has at least 90% sequence identity over

25, preferably over 30 nucleotides forms one aspect of the invention, as does a polynucleotide which has at least 95% sequence identity over 40 nucleotides.

Polynucleotides may be used as a primer, eg a PCR primer or a primer for an  
5 alternative amplification reaction of a probe, eg labelled with a revealing label by  
conventional means for identifying mutations in HM74A and/or HM74 that may be  
implicated in diseases resulting from abnormal lipolysis. Fragments of  
polynucleotides may be fused to the coding sequence of other proteins, preferably  
other G-protein coupled receptors, to form a sequence coding for a fusion protein.

10

Such primers, probes and other fragments will preferably be at least 10,  
preferably at least 15 or at least 20, for example at least 25, at least 30 or at least 40  
nucleotides in length. They will typically be up to 40, 50, 60, 70, 100 or 150  
nucleotides in length. Probes and fragments can be longer than 150 nucleotides in  
15 length, for example up to 200, 300, 400, 500 nucleotides in length, or even up to a  
few nucleotides, such as five or ten nucleotides, short of the coding sequences of  
HM74A and/or HM74.

The polynucleotides have utility in production of HM74A and/or HM74 or  
20 variant polypeptides, which may take place *in vitro*, *in vivo* or *ex vivo*. The  
polynucleotides may be used as therapeutic agents in their own right, in gene therapy  
techniques. The polynucleotides are cloned into expression vectors for these  
purposes. Such expression vectors are routinely constructed in the art of molecular  
biology and may for example involve the use of plasmid DNA and appropriate  
25 initiators, promoters, enhancers and other elements, such as for example  
polyadenylation signals which may be necessary, and which are positioned in the  
correct orientation, in order to allow for protein expression. Other suitable vectors  
would be apparent to a person skilled in the art. By way of further example in this  
regard we refer to Sambrook *et al*.

30 Expression vectors comprise a polynucleotide encoding the desired  
polypeptide operably linked to a control sequence which is capable of providing for  
the expression of the coding sequence by a host cell. The term "operably linked"  
refers to a juxtaposition wherein the components described are in a relationship  
permitting them to function in their intended manner. A regulatory sequence, such as

a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

5       The vectors may be plasmid, virus or phage vectors provided with a origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used *in*  
10 *vitro*, for example for the production of RNA or DNA or used to transfect or transform a host cell, for example, a mammalian host cell. The vectors may also be adapted to be used *in vivo*, for example in a method of gene therapy.

Promoters and other expression regulation signals may be selected to be  
15 compatible with the host cell for which expression is designed. For example, yeast promoters include *S. cerevisiae* GAL4 and ADH promoters, *S. pombe nmt1* and *adh* promoter. Mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium. Viral promoters such as the SV40 large T antigen promoter or adenovirus promoters may also be used. All these  
20 promoters are readily available in the art.

Mammalian promoters, such as  $\beta$ -actin promoters, may be used. Tissue-specific promoters, in particular adipose cell specific promoters are especially preferred. Viral promoters may also be used, for example the Moloney murine  
25 leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art.

30       The vector may further include sequences flanking the polynucleotide which comprise sequences homologous to eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the relevant polynucleotides into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising

the expression cassette flanked by viral sequences can be used to prepare a viral vector suitable for delivering the polynucleotides of the invention to a mammalian cell. Retrovirus vectors for example may be used to stably integrate the polynucleotide into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

Cells are transformed or transfected with the vectors to express the HM74A and/or HM74 polypeptides or a variant thereof. Such cells may be eucaryotic or prokaryotic. They include transient or, preferably, stable higher eukaryotic cell lines such as mammalian cells or insect cells, lower eukaryotic cells such as yeast, and prokaryotic cells such as bacterial cells. Particular examples of cells which may be used to express HM74A and/or HM74 or a variant polypeptide include mammalian HEK293T, CHO, HeLa and COS7 cells. Preferably the cell line selected will be one which is not only stable, but also allows for mature glycosylation and cell surface expression of HM74A and/or HM74 polypeptides or a variant. Cells such as adipocytes expressing HM74A and/or HM74 receptors or a variant polypeptide may be used in screening assays. Expression may be achieved in transformed oocytes. The HM74A and/or HM74 polypeptides or a variant may be expressed in cells such as adipose tissue of a transgenic non-human animal, preferably a rodent such as a mouse.

The present invention is concerned in particular with the use of HM74A and/or HM74 or a functional variant in screening methods to identify agents that may act as modulators of HM74A and/or HM74 receptor activity and, in particular, agents that may act as modulators of lipolysis. Such modulators are useful in the treatment of dyslipidaemia, coronary artery disease, atherosclerosis, obesity and thrombosis, angina, chronic renal failure, peripheral vascular disease, stroke, type II diabetes, inflammation and metabolic syndrome (syndrome X).

Any suitable form of assay may be employed to identify a modulator of HM74A and/or HM74 activity and/or of lipolysis. In general terms, such screening methods involve contacting HM74A and/or HM74 or a variant polypeptide with a test compound and then determining receptor activity. G-protein activation, and especially  $G_i$ -protein activation, may be determined therefore. Where a test



compound affects receptor activity, its effect on lipolysis can be determined by contacting adipocytes in culture with the test compound and measuring lipolysis.

Modulator activity can be determined *in vitro* or *in vivo* by contacting cells  
5 expressing HM74A and/or HM74 or a variant polypeptide with an agent under test and by monitoring the effect mediated by HM74A and/or HM74 or variant polypeptide. Thus, a test agent may be contacted with isolated cells which express HM74A and/or HM74 or a variant polypeptide. The cells may be provided in culture. Cells may be disrupted and cell membranes isolated and used.

10

The HM74A and/or HM74 or variant polypeptide may be naturally or recombinantly expressed. Preferably, an assay is carried out *in vitro* using cells expressing recombinant polypeptide or using membranes from such cells. Suitable eucaryotic and procaryotic cells are discussed above. Preferably adipocytes are used.

15

Typically, receptor activity is monitored by measuring a  $G_i$ -coupled readout.  $G_i$ -coupled readout can be monitored using an electrophysiological method to determine the activity of G-protein regulated  $Ca^{2+}$  or  $K^+$  channels or by using fluorescent dye to measure changes in intracellular  $Ca^{2+}$  levels. Other methods that  
20 can typically be used to monitor receptor activity involved measuring levels of or activity of GTP(S or cAMP).

Yeast assays may be used to screen for agents that modulate the activity of HM74A and/or HM74 or variant polypeptides. A typical yeast assay involves  
25 heterologously expressing HM74A and/or HM74 or a variant polypeptide in a modified yeast strain containing multiple reporter genes, typically FUS1-HIS3 and FUS1-lacZ, each linked to an endogenous MAPK cascade-based signal transduction pathway. This pathway is normally linked to pheromone receptors, but can be coupled to foreign receptors by replacement of the yeast G protein with  
30 yeast/mammalian G protein chimeras. Strains may also contain further gene deletions, such as deletions of SST2 and FAR1, to potentiate the assay. Ligand activation of the heterologous receptor can be monitored for example either as cell growth in the absence of histidine or with a suitable substrate such as beta-galactosidase (lacZ).

Alternatively melanophore assays may be used to screen for activators of HM74A and/or HM74. HM74A and/or HM74 or a variant polypeptide can be heterologously expressed in *Xenopus laevis* melanophores and their activation can be measured by either melanosome dispersion or aggregation. Basically, melanosome dispersion is promoted by activation of adenylate cyclase or phospholipase C, i.e. G<sub>s</sub> and G<sub>q</sub> mediated signalling respectively, whereas aggregation results from activation of G<sub>i</sub>-protein resulting in inhibition of adenylate cyclase. Hence, ligand activation of the heterologous receptor can be measured simply by measuring the change in light transmittance through the cells or by imaging the cell response.

Preferably, control experiments are carried out on cells which do not express HM74A and/or HM74 or a variant polypeptide to establish whether the observed responses are the result of activation of the HM74A and/or HM74 or the variant polypeptide.

Suitable test substances which can be tested in the above assays include combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as display (e.g. phage display libraries) and antibody products. In a preferred embodiment, the test substance is a nicotinic acid (Niacin). Assays may also be carried out using known ligands of other G-protein coupled receptors to identify ligands which act as agonists at HM74A and/or HM74.

Test substances may be used in an initial screen of, for example, 10 substances per reaction, and the substances of these batches which show inhibition or activation tested individually. Test substances may be used at a concentration of from 1nM to 1000μM, preferably from 1μM to 100μM, more preferably from 1μM to 10μM.

Agents which modulate HM74A and/or HM74 activity and which have been identified by assays in accordance with the invention can be used in the treatment or prophylaxis of lipid disorders which are responsive to regulation of HM74A and/or HM74 receptor activity. Agents which activate HM74A and/or HM74 receptor activity and/or which have been identified as inhibitors of lipolysis are preferred. In

particular, such agents may be used in the treatment of dyslipidaemia and conditions associated with dyslipidaemia such as atherosclerosis, obesity, thrombosis or coronary artery disease, angina, chronic renal failure, peripheral vascular disease, stroke, type II diabetes metabolic syndrome (syndrome X) and inflammation.

5

The amount of a HM74A and/or HM74 modulator which is required to achieve the desired biological effect will, of course, depend on a number of factors, for example, the mode of administration and the precise clinical condition of the recipient. In general, the daily dose will be in the range of 0.1mg - 1g/kg, typically  
10 0.1 - 100mg/kg. An intravenous dose may, for example, be in the range of 0.01mg to 0.1g/kg, typically 0.01mg to 10mg/kg, which may conveniently be administered as an infusion of from 0.1µg to 1mg, per minute. Infusion fluids suitable for this purpose may contain, for example, from 0.01µg to 0.1mg, per millilitre. Unit doses may contain, for example, from 0.01µg to 1g of a HM74 modulator. Thus ampoules  
15 for injection may contain, for example, from 0.01µg to 0.1g and orally administrable unit dose formulations, such as tablets or capsules, may contain, for example, from 0.1mg to 1g.

A HM74A and/or HM74 modulator may be employed in the treatment of a  
20 HM74A and/or HM74 mediated disease as the compound per se, but is preferably presented with an acceptable carrier in the form of a pharmaceutical formulation. The carrier must, of course, be acceptable in the sense of being compatible with the other ingredients of the formulation and must not be deleterious to the recipient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the  
25 HM74A and/or HM74 modulator as a unit-dose formulation, for example, a tablet, which may contain from 0.05% to 95% by weight of the HM74 modulator.

The formulations include those suitable for oral, rectal, topical, buccal (e.g. sub-lingual) and parenteral (e.g. subcutaneous, intramuscular, intradermal or  
30 intravenous) administration.

Formulations suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges or tablets, each containing a predetermined amount of a HM74A and/or HM74 modulator; as a powder or granules; as a solution

or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. In general, the formulations are prepared by uniformly and intimately admixing the active HM74A and/or HM74 modulator with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the product. For example, a tablet may be prepared by compressing or moulding a powder or granules of the HM74A and/or HM74 modulator optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the compound in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent and/or surface active/dispersing agent(s). Moulded tablets may be made by moulding, in a suitable machine, the powdered compound moistened with an inert liquid diluent.

Formulations suitable for buccal (sub-lingual) administration include lozenges comprising a HM74A and/or HM74 modulator in a flavoured base, usually sucrose and acacia or tragacanth, and pastilles comprising the HM74A and/or HM74 modulator in an inert base such as gelatin and glycerin or sucrose and acacia.

Formulations of the present invention suitable for parenteral administration conveniently comprise sterile aqueous preparations of an HM74A and/or HM74 modulator, preferably isotonic with the blood of the intended recipient. These preparations are preferably administered intravenously, although administration may also be effected by means of subcutaneous, intramuscular, or intradermal injection. Such preparations may conveniently be prepared by admixing the HM74A and/or HM74 modulator with water and rendering the resulting solution sterile and isotonic with the blood. Injectable compositions according to the invention will generally contain from 0.1 to 5% w/w of the HM74A and/or HM74 modulator.

Formulations suitable for rectal administration are preferably presented as unit-dose suppositories. These may be prepared by admixing a HM74A and/or HM74 modulator with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include vaseline, lanolin, polyethylene glycols, alcohols, and

combinations of two or more thereof. The HM74A and/or HM74 modulator is generally present at a concentration of from 0.1 to 15% w/w of the composition, for example, from 0.5 to 2%.

5           Alternatively agents which up-regulate HM74A and/or HM74 expression or nucleic acids encoding HM74A and/or HM74 or a variant polypeptide may be administered to the mammal. Nucleic acid, such as RNA or DNA, preferably DNA, is provided in the form of a vector, which may be expressed in the cells of a human or other mammal under treatment. Preferably such up-regulation or expression  
10 following nucleic acid administration will enhance HM74A and/or HM74 activity.

Nucleic acid encoding HM74A and/or HM74 or variant polypeptide may be administered to a human or other mammal by any available technique. For example, the nucleic acid may be introduced by injection, preferably intradermally,  
15 subcutaneously or intramuscularly. Alternatively, the nucleic acid may be delivered directly across the skin using a nucleic acid delivery device such as particle-mediated gene delivery. The nucleic acid may be administered topically to the skin, or to the mucosal surfaces for example by intranasal, oral, intravaginal, intrarectal  
administration.

20           Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents includes cationic agents, for example, calcium phosphate and DEAE-Dextran and lipofectants, for example, lipofectam and transfectam. The  
25 dosage of the nucleic acid to be administered can be altered. Typically the nucleic acid is administered in the range of 1pg to 1mg, preferably to 1pg to 10µg nucleic acid for particle mediated gene delivery and 10µg to 1mg for other routes.

Polynucleotides encoding HM74A and/or HM74 or a variant polypeptide can  
30 also be used to identify mutation(s) in HM74 or HM74A genes which may be implicated in human disorders. Identification of such mutation(s) may be used to assist in diagnosis of dyslipidaemia and conditions associated with dyslipidaemia such as, atherosclerosis, obesity, thrombosis, angina, chronic renal failure, peripheral vascular disease, stroke, type II diabetes, inflammation and metabolic syndrome

(syndrome X) or other disorders or susceptibility to such disorders and in assessing the physiology of such disorders.

Antibodies (either polyclonal or preferably monoclonal antibodies, chimeric, single chain, Fab fragments) which are specific for the HM74A and HM74 polypeptides or a variant thereof can be generated. Such antibodies may for example be useful in purification, isolation or screening methods involving immunoprecipitation techniques and may be used as tools to elucidate further the function of HM74A and HM74 or a variant thereof, or indeed as therapeutic agents in their own right. Such antibodies may be used to block ligand binding to the receptors. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of an antibody are well known in the art (see for example Maddox *et al*, J. Exp. Med. 158, 1211 *et seq*, 1993).

The activators, inhibitors, polynucleotides and antibodies for use in the instant invention may be used in combination with one or more other therapeutic agents for example, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins), PPAR modulators (e.g. fibrates) bile acid binding resins, MTP inhibitors and LDL up-regulators. The invention thus provides in a further aspect the use of a combination of a HM74 and/or HM74A modulator (excluding nicotinic acid) and at least one other therapeutic agent, preferably a statin in the treatment of HM74 and/or HM74A mediated disorders.

When the activators, inhibitors and polynucleotides and antibodies are used in combination with other therapeutic agents, the agents may be administered either sequentially or simultaneously by any convenient route.

The combinations referred to above may conveniently be presented for use in the form of a pharmaceutical formulation and thus pharmaceutical formulations comprising a combination as defined above optimally together with a pharmaceutically acceptable carrier or excipient comprise a further aspect of the invention. The individual components of such combinations may be administered either sequentially or simultaneously in separate or combined pharmaceutical formulations.

When combined in the same formulation it will be appreciated that the two components must be stable and compatible with each other and the other components of the formulation and may be formulated for administration. When formulated separately they may be provided in any convenient formulation, conveniently in such a manner as are known for such compounds in the art.

When in combination with a second therapeutic agent active against the same disease, the dose of each component may differ from that when the compound is used alone. Appropriate doses will be readily appreciated by those skilled in the art.

The present invention will now be further illustrated by the accompanying Examples which do not limit the scope of the invention in any way.

#### 15 **Example 1**

To identify possible candidate nicotinic acid receptors, Taqman RNA data was investigated to shortlist 11 orphan receptors which were expressed in adipose and/or omentum together with spleen (Table 1).

20 **Table 1. Orphan GPCRs, which by Taqman mRNA analysis share adipose and/or omental fat and spleen tissue distribution**

Orphan GPCR	Tissue distribution		
	Adipose	Omentum	Spleen
P2Y9	+	+	-
P2Y5	+	+	-
GPR5	+	-	+
GPR52	+	-	+
EBI2	-	+	+
GPR34	-	+	+
HM74	+	+	+
DezB	+	-	+
GPR15	+	-	+
HIPUM 10	+	+	+
HIPUM 15 (EDG8)	-	+	-

Each of the cDNAs for these orphans were then transiently transfected into HEK293T cells together with that of the  $G_i$  family G protein  $G_{o1}\alpha$  and responses measured using [ $^{35}$ S]GTP $\gamma$ S binding following exposure of membranes to nicotinic acid. Fig. 1 shows that only membranes from cells transfected to express the orphan receptor HM74 yielded responses to nicotinic acid. No such responses were measured from any other transfected membranes. Taqman mRNA distribution analysis is depicted in Fig. 2. HM74 mRNA was found to be highly expressed in those tissues commensurate with a nicotinic acid receptor ie spleen, subcutaneous adipose and omental fat.

### Example 2

Further detailed functional analysis of HM74 showed that nicotinic acid elicited a robust, concentration-dependent stimulation of [ $^{35}$ S]GTP $\gamma$ S binding on membranes from HM74-expressing HEK293T cells (Fig. 3). In addition, nicotinuric acid, a structurally related metabolite of nicotinic acid, was not found to alter basal [ $^{35}$ S]GTP $\gamma$ S binding in such membranes (Fig. 4). The  $\alpha$  subunits of the  $G_i$ -like G protein family bind and hydrolyse guanine nucleotides at significantly higher rates than other G protein families such as  $G_q$ ,  $G_s$  and  $G_{12}$ . Therefore, ligand stimulation of [ $^{35}$ S]GTP $\gamma$ S binding is usually indicative of receptor-mediated activation of  $G_i$  G proteins. With the exception of  $G_{2\alpha}$ , the  $\alpha$  subunits of the  $G_i$ -like G proteins all possess a conserved cysteine residue four amino acids from the C-terminus which acts as an acceptor for ADP-ribosylation catalysed by pertussis toxin (3). The addition of the ADP-ribose group serves to prevent GPCR- $G_i$  coupling. Therefore, a simple means of defining whether a cellular response to an agonist at a GPCR is transduced via activation of a  $G_i$ -like G protein is to record attenuation of function following pertussis toxin treatment of cells. Fig. 5 shows that pertussis toxin pre-treatment of HM74 and  $G_{o1}\alpha$ -expressing HEK293T cells (50 ng/ml, 16 h) completely abolished nicotinic acid-mediated stimulation of [ $^{35}$ S]GTP $\gamma$ S binding. These data provide strong evidence that nicotinic acid promotes activation of  $G_i$  G protein signalling via HM74.

To investigate whether HM74 displayed selectivity for any particular  $G_i$ -like G protein, HEK293T cells were co-transfected with HM74 cDNA in combination



with cDNAs for  $G_{o1}\alpha$ ,  $G_{i1}\alpha$ ,  $G_{i2}\alpha$  and  $G_{i3}\alpha$ . The ability of various concentrations of nicotinic acid to stimulate [ $^{35}$ S]GTP $\gamma$ S binding was measured in membranes from transfected cells. Fig. 6 shows that similar concentration-response curves and maximal stimulations were achieved following co-expression of HM74 with each of the  $G_i$ -like G proteins. These data suggest that the nicotinic acid-responsive HM74 receptor has the capacity to interact with all four  $G_i$ -like G proteins and to promote their activation with similar efficiency. The potency of the nicotinic acid response at recombinant HM74 is at least 100-1000-fold right-shifted compared to that measured in native rat adipose and/or spleen which may be due to species receptor variation.

### Example 3

In order to identify species orthologues of HM74, a murine sequence with significant homology to human HM74 was identified by searching public domain databases with the peptide sequence for human HM74 taken from GenBank entry Accession No. D10923. A TBLASTN search produced significant alignments with Accession No.s AJ300198 and AJ300199 describing the *Mus musculus* Puma-g gene for a putative seven transmembrane spanning receptor (termed mHM74A). Noticeably, the encoded peptide sequence had a shorter C-terminal tail when compared to that of the human gene. BESTFIT analysis (Wisconsin Package Version 10.1, Genetics Computer Group (GCG)) showed approx. 81% identity / 84% similarity across the region of ungapped alignment (see Figs.7-9).

Using the murine sequence information, a set of 4 PCR primers were designed to try and amplify regions from the 5' and 3' ends of the corresponding rat gene.

Primer NF454 (5'-ACTGGCCAGATCCACTCATG-3') is identical to the sequence immediately 5' to and including the putative start codon of the murine gene (AJ300198/AJ300199) and was used with primer NF450 (5'-GAACGGCAGGCAGATGATCAG-3') whose sequence is identical to conserved corresponding regions of the coding sequence of D10923 (human HM74) and AJ300198/AJ300199 (murine HM74A). These primers were used in combination to

amplify a fragment of approx. 250bp from rat genomic DNA corresponding to the 5' end of the gene.

This region was amplified using Pfu DNA polymerase under conditions recommended by the manufacturer (*Stratagene*) using an annealing temperature of 50° C, cloned into the vector PCR-Script and sequenced. 3 clones were found to have identical sequence and a new PCR primer (NF459 5'-TAGGATCCACCA**AT**GAGCAAGCAGAACCACTT-3') was designed based on the sequence including and immediately 3' to the putative start codon (underlined in bold). Additional sequence 5' to the start codon was included to encode a BamHI restriction site and a Kozak sequence (believed to help optimise translation).

A 370bp fragment of DNA corresponding to the 3' end of the rat gene was amplified using primers NF455 (5'-ACATGAACAGCATGCTGGAC-3') whose sequence is identical to conserved corresponding regions within the coding sequence of D10923 and AJ300198/AJ300199 and NF458 (5'-GATTCTCCGAATCTAGAAGTTCCA-3') whose sequence is identical to a region 3' to the putative stop codon in AJ300198/AJ300199 and has 21/24 bases in common with a region 3' to the stop codon in D10923.

This region was amplified using Pfu DNA polymerase under conditions recommended by the manufacturer (*Stratagene*) using an annealing temperature of 50° C, cloned into the vector PCR-Script and sequenced. 3 clones were found to have identical sequence. Sequence analysis suggested that the putative stop codon in the rat was in a position analogous to that in the mouse, rather than being further 3' as in the human.

A new PCR primer (NF460 5'-TACTCGAG**T**TAAACGAGATGTGGAAGCCA-3') was designed based on the sequence including and immediately 5' to the putative stop codon. Additional sequence 3' to the stop codon was included to encode a XhoI restriction site.

Primers NF459 and NF460 were used to amplify a fragment containing the entire coding sequence of the rat gene. This region was amplified using Pfu DNA

polymerase under conditions recommended by the manufacturer (*Stratagene*) using an annealing temperature of 55° C, cloned into the vector PCR-Script and sequenced. 3 clones were found to have identical sequence. Full cDNA and translated amino acid sequences are shown in Figs. 10 and 11, respectively.

5

A comparison of the rat and mouse sequences using BESTFIT analysis (Wisconsin Package Version 10.1, Genetics Computer Group (GCG)) showed approx. 95% identity / 97% similarity (Fig. 12).

- 10 A comparison of the rat and human sequences showed approx. 81% identity / 84% similarity (Fig. 13).

- 15 A TBLASTN search of the public domain databases using the rat peptide sequence indicated that there were two regions similar to this in the high throughput draft sequence for homo sapiens chromosome 12 clones RP11-507N20 and RP11-324E6 as represented by GenBank Accession No.s AC026331 and AC026333 respectively. One of these regions represents the same sequence as that present in GenBank Accession No. D10923 (human HM74). The other represents a sequence which shares approximately 96% identity / 96% similarity across the region of ungapped alignment to HM74 (Fig. 14) and which we term HM74A. HM74A would also be similar in length to the mouse and rat sequences that have been described herein.
- 20

- Human HM74A is 83% identical / 86% similar to the mouse gene represented by Accession No.s AJ300198 and AJ300199 (Fig. 15) and is 84% identical / 86% similar to the rat sequence whose isolation is described here (Fig. 16).
- 25

The mouse and rat sequences described here are likely to be the species orthologues of HM74A rather than HM74. HM74 appears to be part of a duplication of a section of human chromosome 12, or its equivalent, in the relatively recent evolutionary past.

#### 5 **Example 4**

Rat HM74A was transfected into HEK293T cells in combination with  $G_{o1}\alpha$  and GTP $\gamma$ S binding measured on membranes in response to nicotinic acid exposure. Parallel experiments were performed using human HM74A. Fig. 17. shows that nicotinic acid stimulated GTP $\gamma$ S binding in both rat and human HM74A-  
10 transfected membranes in a concentration-dependent manner with an approx. EC<sub>50</sub> of 400 nM. This value is very similar to that measured in native adipose and spleen tissue and suggests that HM74A represents a high affinity nicotinic acid receptor. A more detailed pharmacological characterisation was then carried out on rat and human HM74A using a number of nicotinic acid analogues. Fig. 18. shows that rat  
15 (A) and human (B) HM74A display very similar pharmacological profiles with identical rank orders of potency and which are also in good agreement with published data on rat adipose and spleen tissue.

#### 20 **Example 5**

### **METHODS**

#### **Mammalian Cell culture and transfections**

HEK293T cells (HEK293 cells stably expressing the SV40 large T-antigen)  
25 were maintained in DMEM containing 10 % (v/v) foetal calf serum and 2 mM glutamine. Cells were seeded in 60 mm culture dishes and grown to 60-80 % confluency (18-24 h) prior to transfection with pCDNA3 containing the relevant DNA species using Lipofectamine reagent. For transfection, 3  $\mu$ g of DNA was mixed with 10  $\mu$ l of Lipofectamine in 0.2 ml of Opti-MEM (Life Technologies Inc.) and  
30 was incubated at room temperature for 30 min prior to the addition of 1.6 ml of Opti-MEM. Cells were exposed to the Lipofectamine/DNA mixture for 5 h and 2 ml of 20 % (v/v) newborn calf serum in DMEM was then added. Cells were harvested 48-72 h after transfection.

### Preparation of membranes

Plasma membrane-containing P2 particulate fractions were prepared from cell pastes frozen at  $-80^{\circ}\text{C}$  after harvest. All procedures were carried out at  $4^{\circ}\text{C}$ . Cell pellets were resuspended in 1 ml of 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5 (buffer A) and by homogenisation for 20 s with a polytron homogeniser followed by passage (5 times) through a 25-gauge needle. Cell lysates were centrifuged at 1,000 g for 10 min in a microcentrifuge to pellet the nuclei and unbroken cells and P2 particulate fractions were recovered by microcentrifugation at 16,000 g for 30 min. P2 particulate fractions were resuspended in buffer A and stored at  $-80^{\circ}\text{C}$  until required. Protein concentrations were determined using the bicinchoninic acid (BCA) procedure (4) using BSA as a standard.

### High affinity [ $^{35}\text{S}$ ]GTP $\gamma$ S binding.

Assays were performed in 96-well format using a method modified from Wieland and Jakobs, 1994 (5). Membranes (10  $\mu\text{g}$  per point) were diluted to 0.083 mg/ml in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , pH 7.4) supplemented with saponin (10 mg/l) and pre-incubated with 40  $\mu\text{M}$  GDP. Various concentrations of nicotinic acid were added, followed by [ $^{35}\text{S}$ ]GTP $\gamma$ S (1170 Ci/mmol, Amersham) at 0.3 nM (total vol. of 100  $\mu\text{l}$ ) and binding was allowed to proceed at room temperature for 30 min. Non-specific binding was determined by the inclusion of 0.6 mM GTP. Wheatgerm agglutinin SPA beads (Amersham) (0.5 mg) in 25  $\mu\text{l}$  assay buffer were added and the whole was incubated at room temperature for 30 min with agitation. Plates were centrifuged at 1500 g for 5 min and bound [ $^{35}\text{S}$ ]GTP $\gamma$ S was determined by scintillation counting on a Wallac 1450 microbeta Trilux scintillation counter.

### Assays for compound screening

Modulator activity can be determined by contacting cells expressing a polypeptide of the invention with a substance under investigation and by monitoring the effect mediated by the polypeptides. The cells expressing the polypeptide may be *in vitro* or *in vivo*. The polypeptide of the invention may be naturally or recombinantly expressed. Preferably, the assay is carried out *in vitro* using cells expressing recombinant polypeptide. Typically, receptor activity can be monitored indirectly by measuring a Gi-coupled readout.  $G_i$  coupled readout can typically be

monitored using an electrophysiological method to determine the activity of G-protein regulated  $\text{Ca}^{2+}$  or  $\text{K}^{+}$  channels or by using a fluorescent dye to measure changed in intracellular  $\text{Ca}^{2+}$  levels. Other methods that can typically be used to monitor receptor activity involve measuring levels of or activity of  $\text{GTP}\gamma\text{S}$  or cAMP.

5

Preferably, control experiments are carried out on cells which do not express the polypeptide of the invention to establish whether the observed responses are the result of activation of the polypeptide.

10

Mammalian cells, such as HEK293, CHO and COS7 cells over-expressing the protein of choice are generated for use in the assay. Cell lines which may be employed as suitable hosts include i) CHO cells transfected to stably express PLC 2, a PLC isoform which allows  $\text{G}_i$  G proteins to elicit  $\text{Ca}^{2+}$  mobilisation or ii) CHO cells transfected to stably express the  $\text{G}_q$  family G protein  $\text{G}_{16}$  together with a suitable reporter gene e.g. NFAT (nuclear factor activator of T cells). Expression of  $\text{G}_{16}$  permits a wide variety of non- $\text{G}_q$  coupled receptors to mobilise  $\text{Ca}^{2+}$ .

15

96 and 384 well plate, high throughput screens (HTS) are employed using a) fluorescence based calcium indicator molecules, including but not limited to dyes such as Fura-2, Fura-Red, Fluo 3 and Fluo 4 (Molecular Probes); or b) reporter gene read-out Secondary screening involves the same technology. A brief screening assay protocol is as follows:-

20

Mammalian cells stably over-expressing the protein are cultured in black wall, clear bottom, tissue culture coated 96 or 384 well plates with a volume of 100 $\mu\text{l}$  cell culture medium in each well 3 days before use in a FLIPR (Fluorescence Imaging Plate Reader – Molecular Devices). Cells were incubated with 4 $\mu\text{M}$  FLUO-3AM at 30°C in 5% $\text{CO}_2$  for 90 mins and then washed once in Tyrodes buffer containing 3mM probenecid. Basal fluorescence was determined prior to compound additions. Activation results in an increase in intracellular calcium which can be measured directly in the FLIPR.

25

30

The binding of a modulator to a polypeptide of the invention can also be determined directly. For example, a radio labeled test substance can be incubated with the polypeptide of the invention and binding of the test substance to the

polypeptide can be monitored. Typically, the radiolabeled test substance can be incubated with cell membranes containing the polypeptide until equilibrium is reached. The membranes can then be separated from a non-bound test substance and dissolved in scintillation fluid to allow the radioactive content to be determined by scintillation counting. Non-specific binding of the test substance may also be determined by repeating the experiment in the presence of a saturating concentration of a non-radioactive ligand.

#### Yeast assays

Polypeptides can be heterologously expressed in a modified yeast strain containing multiple reporter genes, typically FUS1-HIS3 and FUS1-lacZ, each linked to an endogenous MAPK cascade-based signal transduction pathway. This pathway is normally linked to pheromone receptors, but can be coupled to foreign receptors by replacement of the yeast G protein with yeast/mammalian G protein chimeras. Strains also contain two further gene deletions, of SST2 and FAR1, to potentiate the assay. Ligand activation of the heterologous receptor can be monitored either as cell growth in the absence of histidine or with a substrate of beta-galactosidase (lacZ). This technology is described in WO99/14344.

#### Melanophore assays

Polypeptide of the invention can be heterologously expressed in *Xenopus laevis* melanophores and its activation can be measured by either melanosome dispersion or aggregation. Basically, melanosome dispersion is promoted by activation of adenylate cyclase or phospholipase C *ie* Gs and Gq mediated signalling, respectively, whereas aggregation results from activation of Gi G proteins resulting in inhibition of adenylate cyclase. Hence, ligand activation of the HM74 can be measured simply by measuring the change in light transmittance through the cells or by imaging the cell response.

### CLAIMS

1. A method for identifying agents which modulate the activity of the HM74A and/or HM74 receptor which comprises determining whether the test agent interacts with HM74A and/or HM74.

2. A method according to claim 1 wherein the method is carried out in combination with a ligand for HM74A and/or HM74.

3. A method according to claim 2 wherein the ligand is nicotinic acid.

4. A method of identifying a modulator of HM74A and/or HM74 comprising a test agent with a cell expressing on the surface the receptor HM74 and/or HM74A, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of an agent to said receptor, with an agent to be screened under conditions to permit binding to the receptor; and determining whether the agent binds to, and activates, or inhibits, the receptor, by detecting the presence or absence of a signal generated from the interaction of the compound with the receptor and thereby determines whether the test agent modulates HM74A and/or HM74 activity.

5. A method according to claim 1 - 4 wherein the receptor is selected from human, rodent, murine, rabbit and monkey receptors.

6. A method according to claim 5 wherein the receptor is the rodent, human or murine receptor.

7. A method according to claim 6 wherein the receptor is the human receptor.

8. A method according to any preceding claim wherein the cells are adipocytes.

9. A method according to claim 8 wherein the adipocytes are provided as a differentiated cell line.



10. A method according to claim 8 wherein the adipocytes are primary adipocytes harvested from a human or animal donor.
11. A method according to claims 1 - 7 wherein the second component capable of  
5 providing a detectable signal is a G-protein.
12. A method according to claim 11 wherein the G-protein is G<sub>i</sub>-protein.
13. A test kit suitable for identification of an agent that modulates HM74 activity,  
10 which kit comprises:
- (a) HM74A or HM74 or a variant thereof which is capable of coupling to a G<sub>i</sub>-protein; and
  - (b) means for monitoring HM74 or HM74A activity.
14. A kit according to claim 13 wherein component (a) comprises cells which  
15 express HM74 or HM74A or a said variant thereof.
15. A kit according to claims 13 or 14 wherein component (b) comprises means  
20 for determining whether G<sub>i</sub>-protein is activated.
16. A method for identification of an agent that inhibits lipolysis, which method  
comprises contacting adipocytes *in vitro* with a test agent identified by the method of  
any one of claims 1 to 15 and monitoring lipolysis, thereby determining whether the  
test agent is an inhibitor of lipolysis.
17. An activator of HM74A and/or HM74 activity identified by a method  
25 according to any one of claims 1 or 15, an inhibitor of lipolysis identified by a  
method according to claim 16.
18. An activator according to claim 17 or a polynucleotide which encloses  
30 HM74A and/or HM74 or a variant thereof for use in a method of treatment of the  
human or animal body by therapy.

19. An activator, inhibitor or polynucleotide according to claim 15 for use in the treatment of dyslipidaemia, coronary heart disease, atherosclerosis, thrombosis or obesity, angina, chronic renal failure, peripheral vascular disease, stroke, type II diabetes metabolic syndrome (syndrome X) or inflammation.

5

20. Use of an activator, inhibitor or polynucleotide as defined in claim 18 in the manufacture of a medicament for the treatment of dyslipidaemia, coronary heart disease, atherosclerosis, thrombosis or obesity, angina, chronic renal failure, peripheral vascular disease, stroke, type II diabetes metabolic syndrome (syndrome X) or inflammation.

10

21. Use of a modulator of HM74A and/or HM74 for the manufacture of a medicament for the treatment of HM74A and/or HM74 mediated diseases with the proviso the modulator is not nicotinic acid.

15

22. A method of treating HM74A and/or HM74 mediated disease in an individual which comprises administering to the individual a therapeutic amount of a modulator of HM74A and/or HM74 activity with the proviso the compound is not nicotinic acid.

20

23. The use of a combination of a HM74 and/or HM74A modulator (excluding nicotinic acid) and at least one other therapeutic agent in the treatment of HM74 and/or HM74A mediated disorders.

25

24. Use according to claim 23 wherein the further therapeutic agents include a statin

Fig. 1.

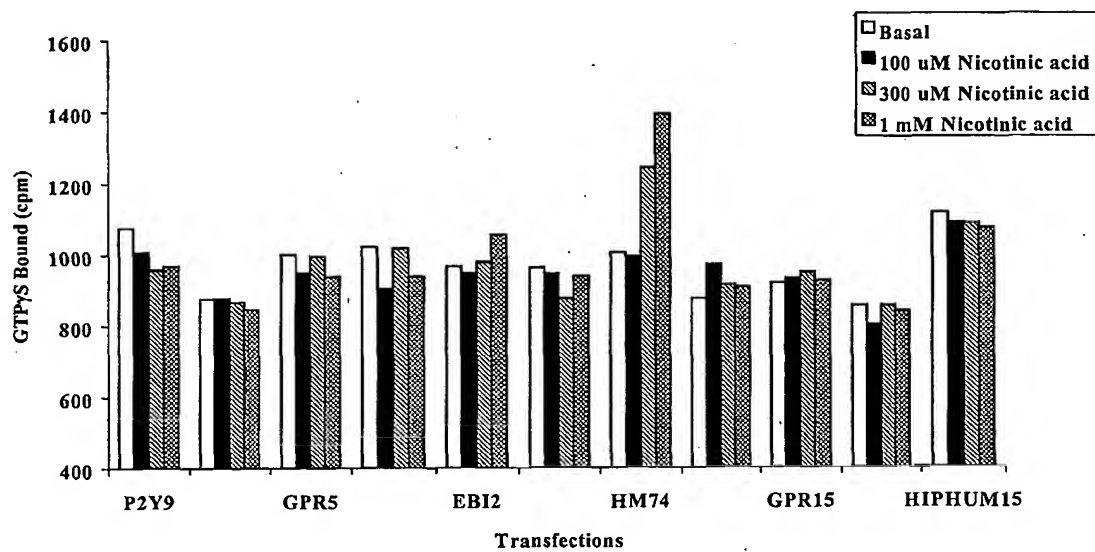
HEK293T stimulation of [ $^{35}$ S]

Fig. 2.

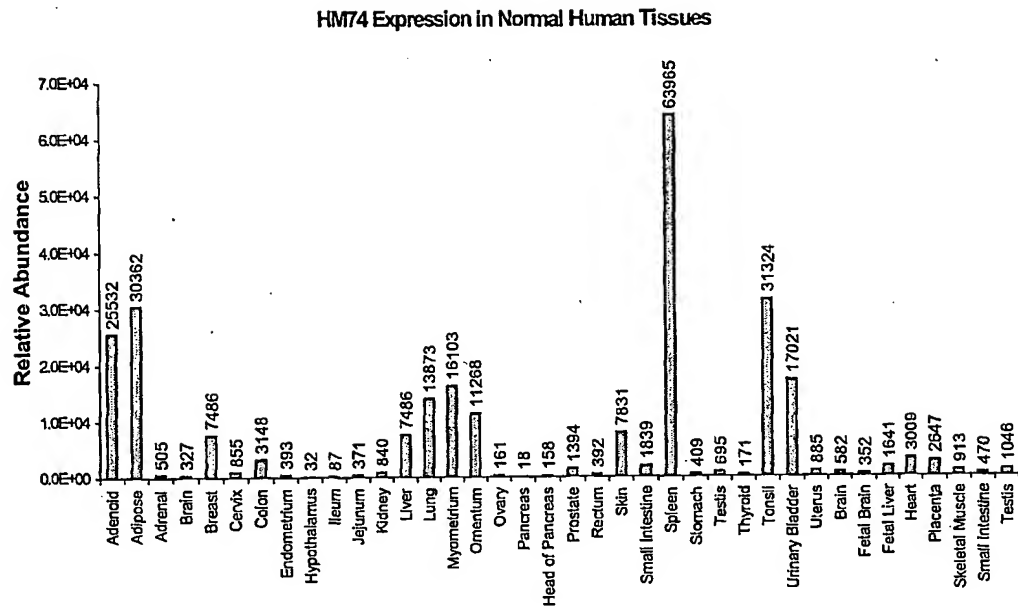


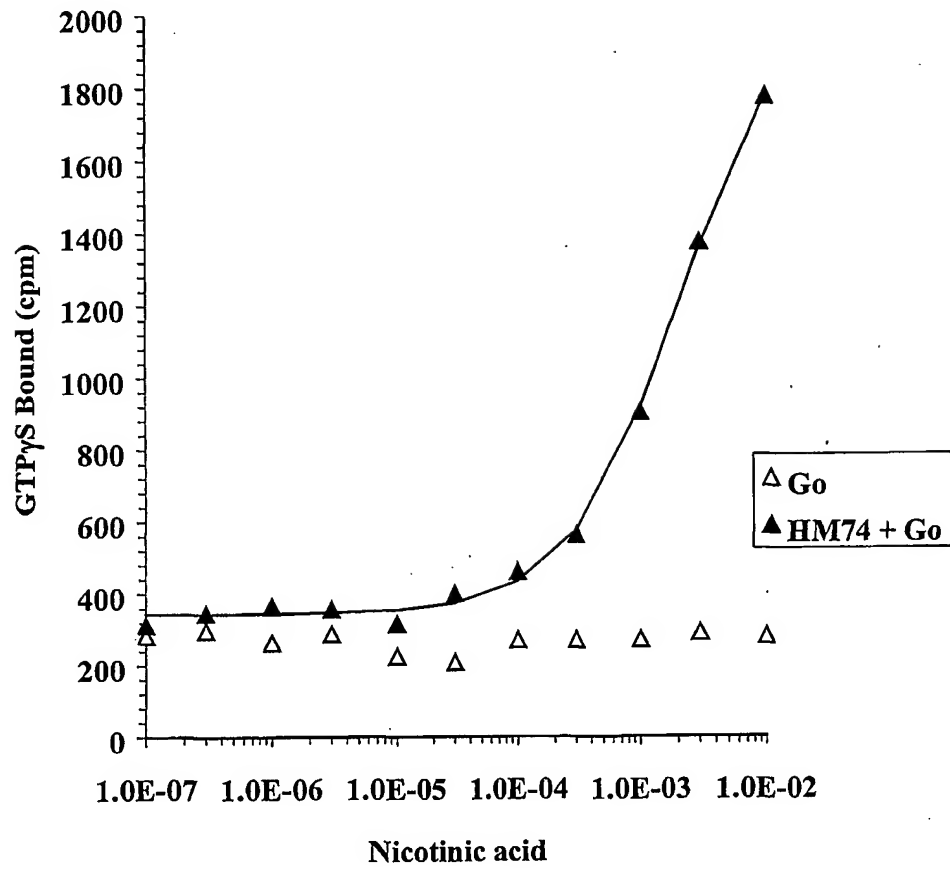
Fig. 3. [ $^{35}$ S]GTP $\gamma$ S binding in membranes

Fig. 4.

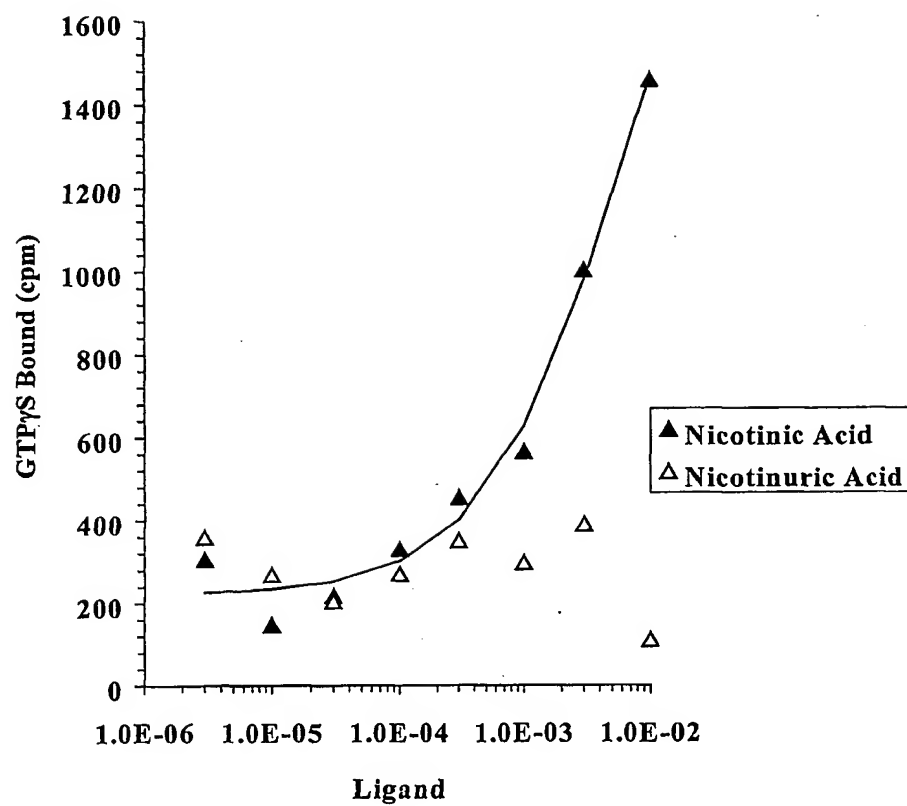


Fig. 5.

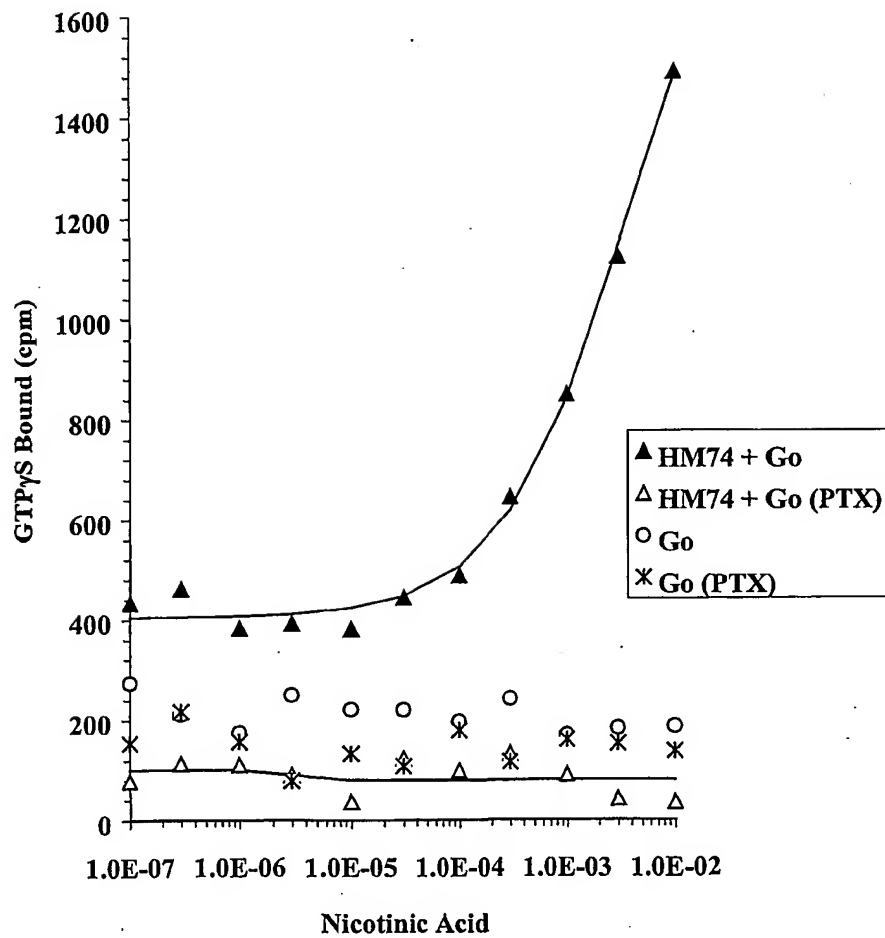


Fig. 6.

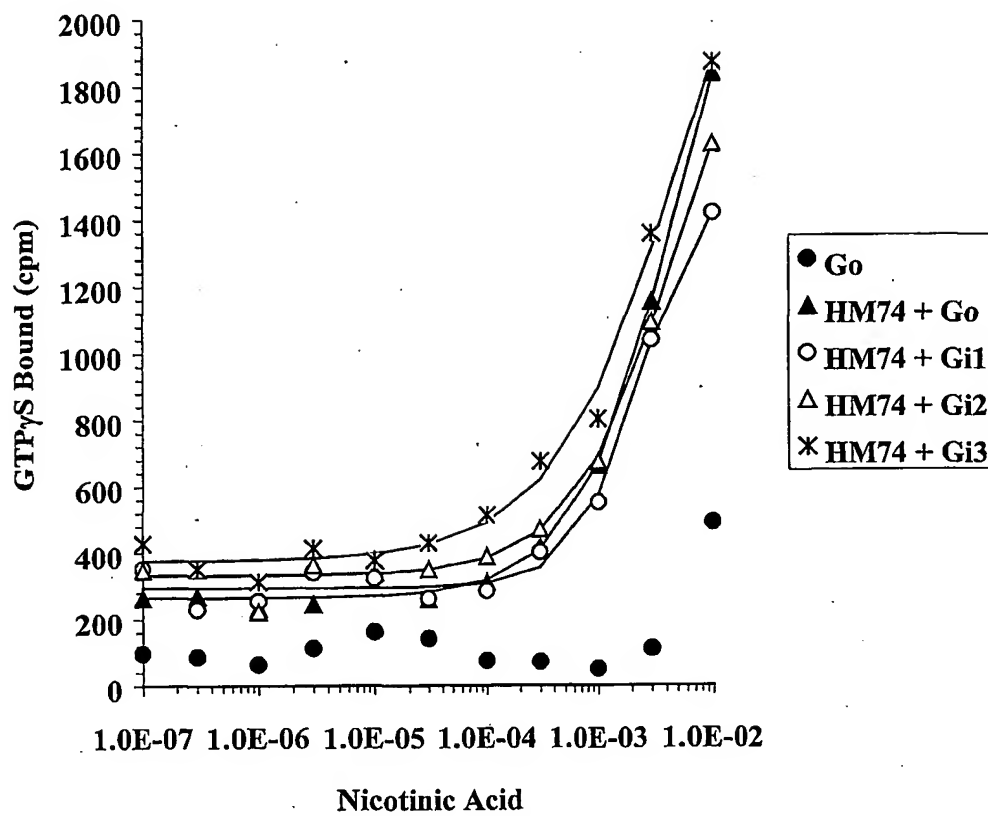




Fig.7. Nucleotide sequence of murine HM74A

ATGAGCAAGTCAGACCATTTTCTAGTGATAAACGGCAAGAACTGCTGTGTGTTCCGAGAT  
 60  
 5 GAAAACATCGCCAAGGTCTTGCCACCGGTGTTGGGGCTGGAATTTGTGTTCCGACTCCTG  
 120  
 GGCAATGGCCTTGCTTGTGGATTTTCTGTTTCCACCTCAAGTCTGGAAATCCAGCCGG  
 180  
 ATTTTCTTGTTCAACTTGGCCGTGGCTGACTTCTCCTGATCATCTGCCTGCCGTTCTCTG  
 240  
 10 ACGGACAACATATGTCCATAACTGGGACTGGAGGTTCCGAGGCATCCCTTGCCGTGTGATG  
 300  
 CTCTTCATGTTGGCTATGAACCGACAGGGCAGCATCATCTTCCTCACCGTGGTGGCTGTG  
 360  
 GACCGCTACTTCCGGGTGGTCCATCCACACCACTTCTTGAACAAGATCTCCAACCGGACG  
 420  
 15 GCGGCCATCATTTCTTGCTTCTTGTGGGGTCTCACCATCGGCCTGACTGTCCACCTCCTC  
 480  
 TATACAAACATGATGACCAAAAATGGCGAGGCATATCTGTGTAGCAGCTTCAGCATCTGT  
 540  
 20 TACAACCTCAGGTGGCAGCATGCTATGTTCTCTTGAATTCCTTCTTGCCCCTGGCCATC  
 600  
 ATCTTGTTCTGCTCAGGCAGGATCATCTGGAGCCTGAGGCAGAGACAGATGGACAGACAT  
 660  
 25 GCCAAGATCAAGAGGGCCATCAACTTCATCATGGTGGTGGCTATTGTATTTCATCATTTGC  
 720  
 TTCTTACCCAGTGTGGCTGTGCGCATCCGCATCTTCTGGCTTCTCTACAAATATAACGTA  
 780  
 CGCAACTGTGACATCTACTCCTCGGTGGACCTGGCTTCTTTACCACCTTAGCTTTTACC  
 840  
 30 TACATGAACAGCATGCTGGACCCTGTGGTCTACTATTTCTCCAGCCCATCTTTCCCCAAC  
 900  
 TTCTTCTCCACGTGTATCAACCGCTGCCTTCGAAAGAAAACATTGGGTGAACCCGATAAT  
 960  
 AACCGAAGCACTAGTGTGGAGCTCACGGGGGACCCAGCACACCAGAAGTATTCCAGGG  
 1020  
 35 GCGCTAATGGCTGACCCCAGTGAGCCAGGCAGCCCCCTTATCTGGCTTCCACATCTCGT  
 1080  
 TAA  
 1083  
 40

Fig.8. Amino acid sequence of murine HM74A

MSKSDHFLVI NGKNCCVFRD ENIAKVLPPV LGLEFVFGLL GNGLALWIFC 50  
 FHLKSWKSSR IFLFNLAVID FLLIICLPFL TDNYVHNWDW RFGGIPCRVM 100  
 LFMLAMNRQG SIIFLTVVAV DRYFRVHHPH HFLNKISNRT AAIISCFLWG 150  
 45 LTIGLTVHLL YTNMMTKNGE AYLCSFSIC YNFRWHDAMF LLEFFLPLAI 200  
 ILFCSGRIIW SLRQRQMDRH AKIKRAINFI MVVAIVFIIC FLPSVAVRIR 250  
 IFWLLYKYNV RNCDIYSSVD LAFFTLSFT YMNSMLDPVV YFSSPSFPN 300  
 FFSTCINRCL RKKTLGEPDN NRSTSVELTG DPSTTRSIPG ALMADPSEPG 350  
 SPPYLASTSR 360  
 50



Fig.10. cDNA sequence of rat HM74A

ATGAGCAAGCAGAACCACTTTCTGGTGATAAACGGCAAGAACTGCTGTGTGTTCCGAGAT  
 60  
 GAAACATCGCCAAGGTCCTGCCGCCGGTGTGGGGCTGGAGTTTGTGTTTGGACTCCTG  
 5 120  
 GGTAATGGCCTTGCCCTGTGGATCTTCTGTTTCCATCTCAAATCCTGGAAATCCAGCCGG  
 180  
 ATTTTCTTGTTCAACCTGGCCGTGGCTGACTTTCTCCTGATCATTTGCTTGCCGTTCTTG  
 240  
 10 ACGGACAACATATGTCCAGAACTGGGACTGGAGGTTCCGGGAGCATCCCCTGCCGCGTGATG  
 300  
 CTCTTCATGTTGGCCATGAACCGACAGGGCAGCATCATCTTCCTCACGGTGGTGGCTGTG  
 360  
 GACAGGTACTTCAGGGTGGTCCACCCGCACCACTTCCTGAACAAGATCTCCAACCGGACG  
 15 420  
 GCGGCCATCATCTCTTGCTTCCTGTGGGGCATCACCATCGGCCTGACAGTCCACCTCCTC  
 480  
 TACACGGACATGATGACCCGAAACGGCGATGCAAACCTGTGCAGCAGTTTAGCATCTGC  
 540  
 20 TACACTTTCAGGTGGCAGATGCAATGTTCTCTTGGAATTCTTCCTGCCCTGGGCATC  
 600  
 ATCCTGTTCTGCTCTGGCAGGATCATTTGGAGCCTAAGGCAGAGACAGATGGACAGGCAC  
 660  
 GTCAAGATCAAGAGGGCCATCAACTTCATCATGGTGGTTGCCATTGTGTTTGTTCATCTGC  
 25 720  
 TTCTTGCCCAGTGTGGCCGTGAGGATCCGCATCTTCTGGCTCCTCTACAAACACAACGTG  
 780  
 AGGAACTGTGACATCTACTCCTCTGTGGACTTGGCCTTCTTACCACCCTTAGCTTTACC  
 840  
 30 TACATGAACAGCATGCTCGACCCGGTGGTCTACTATTTCTCCAGCCCATCTTCCCCAAC  
 900  
 TTCTTCTCCACGTGCATCAACCGTTGCCTTCGAAGGAAAACCTTGGGCGAACCAGATAAT  
 960  
 AACCGGAGCACGAGTGTGGAGCTCACGGGGGACCCCAGCACAAATCAGAAGTATTCAGGG  
 35 1020  
 GCATTAATGACTGACCCAGTGAGCCAGGCAGCCCCCTTATCTGGCTTCCACATCTCGT  
 1080  
 TAA  
 1083  
 40

Fig. 11. Amino acid sequence of rat HM74A

MSKQNHFLVI NGKNCCVFRD ENIAKVLPPV LGLEFVFGLL GNGLALWIFC 50  
 FHLKSWKSSR IFLFNLAAD FLIIICLPFL TDNYVQNWWD RFGSIPCRVM 100  
 LFMLAMNRQG SIIFLTVVAV DRYFRVHPPH HFLNKKISNRT AAIISCFLWG 150  
 45 ITIGLTVHLL YDMMTRNGD ANLCSSFSIC YTFRWHAMF LLEFFLPLGI 200  
 ILFCSGRIIW SLRQRQMDRH VKIKRAINFI MVVAIVFVIC FLPSVAVRIR 250  
 IFWLLYKHNV RNCDIYSSVD LAFFTTLSFT YMNSMLDPVV YFSSPSFPN 300  
 FFSTCINRCL RRKTLGEPDN NRSTSVELTG DPSTIRSIPG ALMTDPSEPQ 350  
 SPPYLASTSR 360  
 50



Fig. 13. Alignment of rat HM74A and human HM74 amino acid sequences

BESTFIT of: Rat HM74A (peptide) from 1 to 360  
to: Human HM74 (peptide) from 1 to 387

```

5          Gap Weight:      8          Average Match:  2.912
          Length Weight:    2          Average Mismatch: -2.003

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          Ratio:      4.329          Gaps:      0
10  Percent Similarity: 83.989  Percent Identity: 81.180

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          . = 1
15

          4 QNHFLVINGKNCCVFRDENIAKVLPPVLGLEFVFGLLGNGLALWIFCFHL 53
          |.||| |. ||||| |: ||||| |: ||||| |: |||||
20          7 QDHFLEIDKKNCVFRDDFIAKVLPPVLGLEFIFGLLGNGLALWIFCFHL 56

          54 KSWKSSRIFLFNLAVADFLLIICLPFLTDNYVQNWDRFGSIPCRVMLFM 103
          ||||| ||||| ||||| . ||| . || |||||
          57 KSWKSSRIFLFNLAVADFLLIICLPFVMDYYVRRSDWNFGDIPCRVLVLFM 106
25          104 LAMNRQGSIIFLTVAVDYFRVVPHHFLNKNISNRTAAIISCFWLGITI 153
          ||||| ||||| ||||| ||||| ||||| ||||| :
          107 FAMNRQGSIIFLTVAVDYFRVVPHHALNKNISNWTAAIISCLWGITV 156

          154 GLTVHLLYTDMTRNGDANLCSSFSICYTFRWHDAMFLEFFLPLGIILF 203
          ||||| :. || ||| : ||||| : ||||| : |||||
          157 GLTVHLLKKLLIQNGPANVCISFSICHTFRWHEAMFLEFFLPLGIILF 206

          204 CSGRIIWSLRQRMDRHVKIKRAINFIMVVAIVFVICFLPSVAVRIRIFW 253
          || ||||| ||||| ||||| ||||| ||||| |||||
35          207 CSARIIWSLRQRMDRHAKIKRAITFIMVVAIVFVICFLPSVVVRIRIFW 256

          254 LLYKHNVNRNCDIYSSVDLAFFTTLSFTYMNSMLDPVYYYFSSPSFPNFFS 303
          ||: . ||: ||||| ||||| ||||| ||||| |||||
40          257 LLHTSGTQNCVYRSVDLAFFITLSFTYMNSMLDPVYYYFSSPSFPNFFS 306

          304 TCINRCLRRKTLGEPDNNRSTSVELTGDPSTIRSIPGALMTDPSEPGSPP 353
          | |||||. || ||||| ||||| . | ||| . || ||
          307 TLINRCLQRKMTGEPDNNRSTSVELTGDPNKTTRGAPEALMANSGEPSWSPS 356
45          354 YLASTS 359
          || ||
          357 YLGPTS 362
50

```

Fig. 14. Alignment of human HM74 and HM74A amino acid sequences

BESTFIT of: Human HM74A (peptide) 1 to 363  
to: Human HM74 (peptide) 1 to 387

5           Gap Weight:       8       Average Match: 2.912  
          Length Weight:     2       Average Mismatch: -2.003

          Quality:   1844           Length:    362  
          Ratio:    5.094           Gaps:     0

10   Percent Similarity: 96.133   Percent Identity: 95.580

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15

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1  MNRHHLQDHFLEIDKKNCCVFRDDFIVKVLPPVLGLEFIFGLLGNGLALW 50
  ||||||||||||||||||||||||||||||||||||||||||||||||
20 1  MNRHHLQDHFLEIDKKNCCVFRDDFIAKVLPPVLGLEFIFGLLGNGLALW 50
  ||||||||||||||||||||||||||||||||||||||||||||||||

51  IFCFHLKSWKSSRIFLFNLAVADFLLIICLPFLMDNYVRRWDWKFGDIPC 100
  |||||||||||||||||||||||||||||||||||||||||||||.|||
51  IFCFHLKSWKSSRIFLFNLAVADFLLIICLPFVMDYYVRRSDWNFGDIPC 100
  |||||||||||||||||||||||||||||||||||||||||||||.|||

25 101 RLMLFMLAMNRQGSIIFLTVAVDTRYFRVVHPHALNKISNRTAAIISCL 150
  |||.||| ||||||||||||||||||||||||||||||||||||||||
101 RLVLFMFAMNRQGSIIFLTVAVDTRYFRVVHPHALNKISNWTAAIISCL 150
  |||.||| ||||||||||||||||||||||||||||||||||||||||

30 151 LWGITIGLTVHLLKKKKMPIQNGGANLCSFSICHTFQWHEAMFLEFFLP 200
  |||||:|||||:||||| |||.||| |||||||||.|||||||
151 LWGITVGLTVHLLKKLLIQNGPANVCISFSICHTFRWHEAMFLEFFLP 200
  |||||:|||||:||||| |||.||| |||||||||.|||||||

35 201 LGIILFCSARIIWSLRQROMDRHAKIKRAITFIMVVAIVFVICFLPSVVV 250
  ||||||||||||||||||||||||||||||||||||||||||||
201 LGIILFCSARIIWSLRQROMDRHAKIKRAITFIMVVAIVFVICFLPSVVV 250
  ||||||||||||||||||||||||||||||||||||||||||||

40 251 RIRIFWLLHTSGTQNCVYRSVDLAFFITLSFTYMNSMLDPVYYFSSPS 300
  ||||||||||||||||||||||||||||||||||||||||||||
251 RIRIFWLLHTSGTQNCVYRSVDLAFFITLSFTYMNSMLDPVYYFSSPS 300
  ||||||||||||||||||||||||||||||||||||||||||||

301 FPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANS 350
  ||||||||||||||||||||||||||||||||||||||||||||
301 FPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANS 350
  ||||||||||||||||||||||||||||||||||||||||||||

45 351 EPWSPSYLGPTS 362
  ||||||||
351 EPWSPSYLGPTS 362
  ||||||||

```

50

BESTFIT of: Human HM74A (peptide) from 1 to 363  
to: Mouse HM74A (peptide) from 1 to 360

```

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      Length Weight:    2      Average Mismatch: -2.003

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10     Percent Similarity: 86.197      Percent Identity: 83.099

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15     . = 1

      8 DHFLEIDKKNCVFRDDFIVKVLPPVLGLEFIFGLLGNGLALWIFCFHLK 57
      |||| |. ||||| |: ||||| |: ||||| |||||
20     5 DHFLVINGKNCCVFRDENIAKVLPPVLGLEFVFGLLGNGLALWIFCFHLK 54
      58 SWKSSRIFLFNLAVADFLLIICLPFLMDNYVRRWDWKFGDIPCRMLFML 107
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
25     55 SWKSSRIFLFNLAVADFLLIICLPFLTDNYVHNWDWRFGGIPCRVMLFML 104
      108 AMNRQGSIIFLTTVVAVDRYFRVVHPPHHLNKKISNRTAAIISCLLWGITIG 157
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
30     105 AMNRQGSIIFLTTVVAVDRYFRVVHPPHHLNKKISNRTAAIISCFWLGLTIG 154
      158 LTVHLLKKKMPIQNGGANLCSFSICHTFQWHEAMFLEFFLPLGIILFC 207
      ||||| |. || | ||||| |: |. |: ||||| ||||| |||||
35     155 LTVHLLYTNNMTKNGEAYLCSSFSICYNFRWHDAMFLEFFLPLAILFC 204
      208 SARIIWSLRQRQMDRHAKIKRAITFIMVVAIVFVICFLPSVVVRIRIFWL 257
      | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
40     205 SGRIIWSLRQRQMDRHAKIKRAINFIIMVVAIVFIICFLPSVAVRIRIFWL 254
      258 LHTSGTQNCDEVYRSVDLAFFITLSFTYMNMSMLDPVVVYFSSPSFPNFFST 307
      |: . ||:: ||||| ||||| ||||| ||||| ||||| ||||| |||||
45     255 LYKYNVRNCDIYSSVDLAFFTTLSFTYMNMSMLDPVVVYFSSPSFPNFFST 304
      308 LINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANSGEPPWSPSY 357
      |||||. : ||||| ||||| ||||| |||||. || |||||. || |||
50     305 CINRCLRKKTGEPDNNRSTSVELTGDPTSTRSIPGALMADPSEPGSPPY 354
      358 LGPTS 362
      | |
55     355 LASTS 359

```

Fig. 16. Amino acid sequence alignment of human HM74A and rat HM74A

BESTFIT of: Human HM74A (peptide) from 1 to 363

to: Rat HM74A (peptide) from 1 to 360

5           Gap Weight:       8           Average Match: 2.912  
             Length Weight:   2          Average Mismatch: -2.003

                  Quality:   1591                   Length:    356  
                   Ratio:    4.469                  Gaps:       0

10   Percent Similarity: 85.955   Percent Identity: 83.708

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                           . = 1

15

7 QDHFLEIDKKNCVFRDDFIVKVLPPVLGLEFIFGLLGNGLALWIFCFHL 56  
 |.|||.|.|||||||:| |||||||||:|||||||

20   4 QNHFLVINGKNCCVFRDENIAKVLPPVLGLEFVFGLLGNGLALWIFCFHL 53  
 |||||||

57 KSWKSSRIFLFLNLAVADFLLIICLPFLMDNYVRRWDWKFGDIPCRMLLFM 106  
 |||||||

54 KSWKSSRIFLFLNLAVADFLLIICLPFLTDNYVQNWDRFGSIPCRVMLFM 103  
 |||||||

25   107 LAMNRQGSIIFLTVAVDYFRVVPHPHALNKISNRTAAIISCLLWGITI 156  
 |||||||

104 LAMNRQGSIIFLTVAVDYFRVVPHPHFLNKISNRTAAIISCLFLWGITI 153  
 |||||||

30   157 GLTVHLLKKKMPIQGGANLCSFSICHTFQWHEAMFLEFFLPLGIILF 206  
 |||||||

154 GLTVHLLYTDMMTRNGDANLCSFSICYTFRWHDAMFLEFFLPLGIILF 203  
 |||||||

207 CSARIISLQRQMDRHAKIKRAITFIMVVAIVFVICFLPSVVVRIRIFW 256  
 || |||||||

35   204 CSGRIISLQRQMDRHVKIKRAINFIMVVAIVFVICFLPSVAVRIRIFW 253  
 || |||||||

257 LLHTSGTQNCVYRSVDLAFFITLSFTYMNSMLDPVVYYFSSPSFPNFFS 306  
 ||: .||:| |||||||

40   254 LLYKHNVNRNCDIYSSVDLAFFITLSFTYMNSMLDPVVYYFSSPSFPNFFS 303  
 ||: .||:| |||||||

307 TLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANSGEPSWSPS 356  
 | |||||.|| |||||||

304 TCINRCLRRKTLGEPDNNRSTSVELTGPSTIRSIPGALMTDPSEPGSPP 353  
 | |||||.|| |||||||

45   357 YLGPTS 362  
       || ||

354 YLASTS 359



Fig 17.

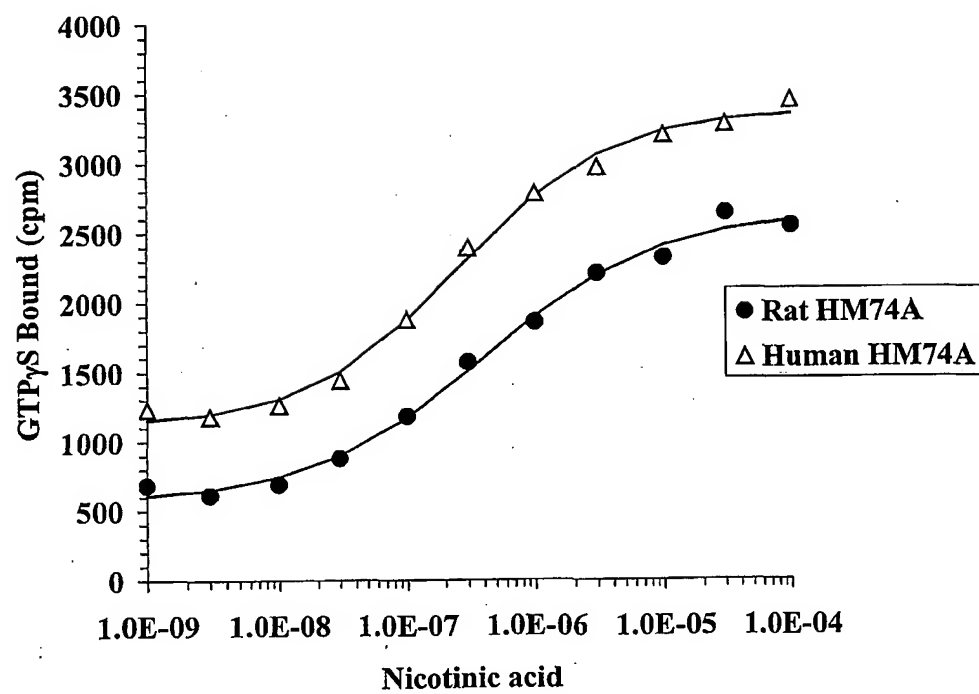


Fig. 18A.

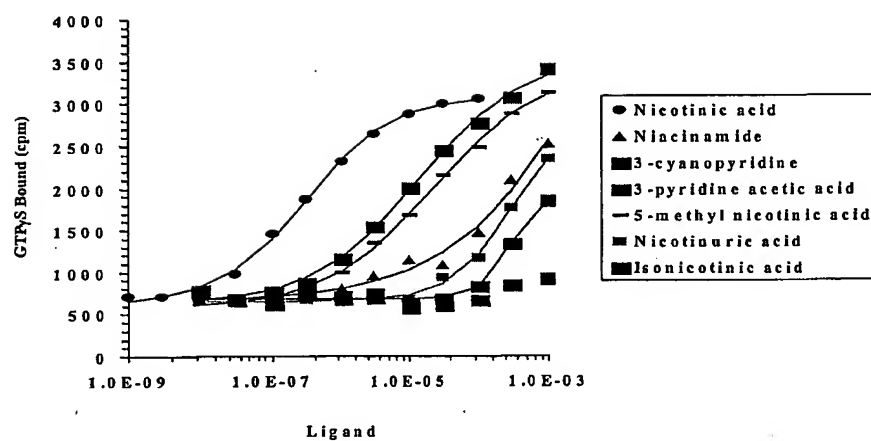
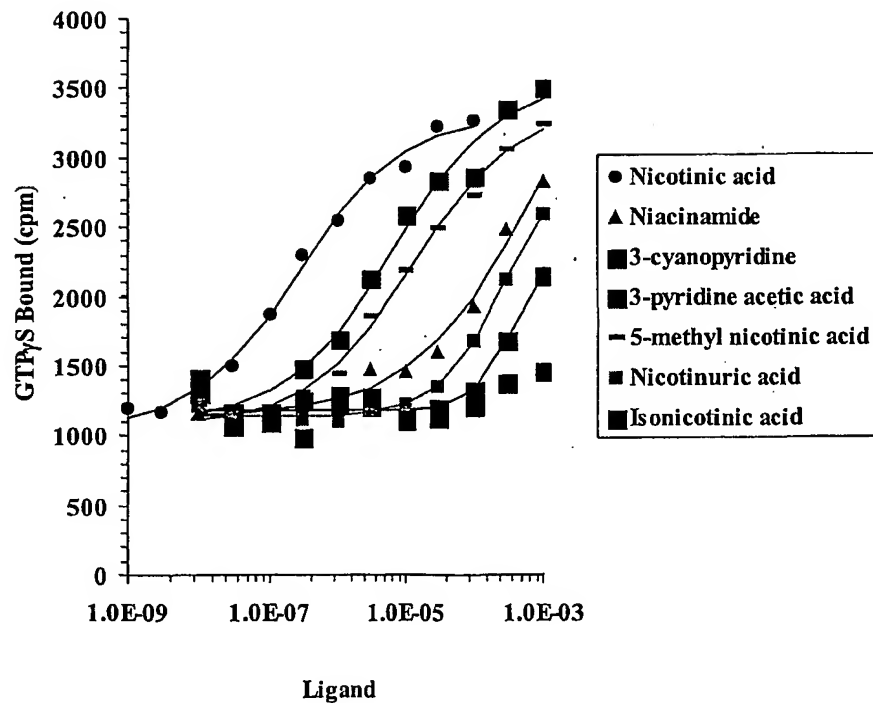


Fig. 18B



**Fig. 19. cDNA sequence for monkey HM74A receptor:**

ATGAATCGGCACCATCTGCAGGATCACTTTCTGGTAATAGACAAGA  
AGAACTGCTGTGTG

TTCCGAGATGACTTCATTGTCAAGGTGTTGCCGCCGGTGTGGGG  
CTGGAGTTTATCTTC

GGGCTTCTGGGCAATGGCCTTGCCCTGTGGATTTTCTGTTTCCAC  
CTCAAGTCCTGGAAA

TCCAGCCGGATTTTCCTGTTCAACCTGGCAGTGGCTGACTTTCTCC  
TGATCATCTGCCTG

CCATTCTGATGGACAACATATGTGAGACGTTGGGACTGGAAGTTT  
GGGGACATCCCTTGC

CGGCTGATGCTCTTCATGCTGGCCATGAACCGCCAGGGCAGCATC  
ATCTTCCTCACGGTG

GTGGCCGTGGACAGGTATTTCCGGGTTGTCCATCCCCACCACGCC  
CTGAACAAGATCTCC

AATCGAACAGCAGCCATCATCTCTTGCCCTTCTGTGGGGTGTCACTA  
TTGGCCTGACAGTC

CACCTCCTGAAGAGGAAGATGCCGATCCAGAATGGCACTGCGAAT  
CTGTGCAGCAGCTTC

AGCATCTGCAATACCTTCCGGTGGCACGAAGCCATGTTCTCCTG  
GAGTTCTTCCTGCCC

CTGGGCATCATCCTGTTCTGCTCAGCCAGAATTATCTGGAGCCTG  
CGGCAGAGACAAATG

GACCGGCATGCCAAGATCAAAAGAGCCATCACCTTCATCATGGTC  
GTGGCCATCGTCTTT

GTCATCTGCTTCCTTCCCAGCGTGGCTGTGCGGATACGCATCTTC  
TGGCTCCTGCACACT

TTTGGCACGCAGAACTGTGAAGTGTACCGCTCGGTGGACCTGGCG  
TTCTTTATCACTCTC

AGTTTTCACCTACATGAACAGCATGCTGGACCCCGTGGTGTACTACT  
TCTCCAGCCCATCC

TTTCCCAACTTCTTCTCCACTTTGATCAACCGCTGCCTCCGAAGGA  
AGATGACAGGTGAC

CCAGATAATAACCGCAGCACGAGTGTGAGCTCACGGGGGACCC  
GAACACAACCAGAGGC

GCTCCAGAGGCATTAATGGCCAACCCAGTGAGCCATGGAGCCC  
CTCTTATCTGGGTCCA  
ACCTCTCGTTAA

**Fig. 20. Peptide sequence for Monkey HM74A receptor:**

MNRHHLQDHFLVIDKKNCCVFRDDFIVKVLPPVLGLEFIFGLLGNGLAL  
WIFCFHLKSWK

SSRIFLNLAVADFLLIICLPFLMDNYVRRWDWKFGDIPCRLMLFMLAM  
NRQGSIIFLTV

VAVDYFRVPHPHALNKISNRTAAIISCLLWGVITIGLTVHLLKRKMPI  
QNGTANLCSSF

SICNTFRWHEAMFLEFFLPLGIILFCSARIWSLRQRQMDRHAKIKRAI  
TFIMVVAIVF

VICFLPSVAVRIRIFWLLHTFGTQNCVYRSVDLAFFITLSFTYMNSML  
DPVYYYFSSPS

FPNFFSTLINRCLRRKMTGDPDNNRSTSVELTGPNTTRGAPEALMA  
NPSEPWSPSYLGP  
TSR

**Fig. 21: Nucleic acid sequence for rabbit HM74A**

GCTAGTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTGCCCTTT  
GCGCGGCCCCAGTCTGGTCTCCCCATGAACCAGCACCGCCCGCAGAGCCATTTTCTGGAG  
ATAGACAAGAAGAACTGCTGCGTGTTCGAGATGACTTCATCGCCAACGTGCTGCCGCCC  
GTGCTGGGGCTGGAGTTCGTGTTCGGGCTGCTGGGCAATGGCCTCGCCTTGTGGATTTTC  
TGCTTCCACCTCAAGTCCTGGAATCCAGCCGGATTTTCCTGTTCAACCTGGCCGTGGCTG  
ACTTCCTCCTGATCATTTGCCTGCCCTTCCTGACGGACAACCTATATGAGGAAGTGGGATT  
GGAGGTTTCGGGGACATCCCATGCCGGCTGATGCTCTTCATGCTGGCCATGAACCGCCAGG  
GCAGCATCATCTTCCTCACGGTGGTCGCCGTGGACAGGTAATTCGGGTGGTCCACCCTC  
ACCACGCCCTGAACAAGATCTCCAACCGGACAGCGGCCGCCATCTCCTGCCTGCTGTGGG  
GCGTCACCATCGGCCTGACGGTCCACCTCCTGCGCAAAGGATGCTGACCCAGAACGGC  
CCGGCCAATCTGTGCAGCAGCTTCAGCATCTGCAACACCTTCGGGTGGCAGCAGCCATG  
TTCCTTCTGGAGTTCCTTCCTGCCCCCTCGCCATCATCTCTTCTGCTCCGTCCGGATCGTCTG  
GAGCCTGCGGCAGAGGCAGATGGACAGGCACGTCAAGATCAAGAGGGCCATCAACTTCA  
TCATGGTGGTGGCCGTGGTCTTCATCATCTGCTTCCTGCCAGCGTGGCCGTGCGGATGC  
GTATCTTCTGGCTCCTGCGCACGGCGGGGACGCAGGACTGTGACGTGTACCGCTCCGTGC  
ACCTGGCCTTCTTCATCACCTCAGCTTACCTACATGAACAGCATGCTGGACCCCTGGT  
CTACTACTTCTCCAGCCCCTCGTTCCCCAACTTCTTCTCCGCGCTCATCAACCGCTGCCTG  
CGGAGGAGCCAGCAGGTGAGCCGGAGAACACAGGAGCACCAGCGTGGAGCTCACTG  
GGGACCCGAGCACCCTCGGAGCGCTCCGGACGCGCTAGTGGCCGAGCCCAACGGGCCA  
CGGAGCCCCTCCTACCTGGTCCCAAATCCTCGTTAGACGGTGGTTCGGAGGAAGGAGACT  
GTCGCAAAGGGCAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTA

**Fig. 22: Nucleic acid sequence for rabbit HM74A**

MNQHRPQSHFLEIDKKNCCVFRDDFIANVLPPVLGLEFVFGLLGNGLALWIFCFHLKSWKSSR  
IFLFNLAVADFLLIICLPFLTDNYMRKWDWRFGDIPCLMLFMLAMNRQGSIIFLTVAVDY  
FRVVHPHHAHNKISNRTAAAIISCLWGVITGLTVHLLRKRMLTQNGPANLCSSFSICNTFRWH  
DAMFLEFFLPLAILFCSVRIVWSLRQRQMDRHVKIKRAINFMVVAVVFIICFLPSVAVRMRI  
FWLLRTAGTQDCDVYRSVDLAFFITLSFTYMNSMLDPLVYYFSSPSFPNFFSALINRCLRRSPA  
GEPENNRSTSVELTGDPSTARSAPDALVAEPNGPRSPSYLVPNPR

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- (72) Inventors; and
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- (74) Agent: **GIDDINGS, Peter, John**; Corporate Intellectual Property, GlaxoSmithKline, 980 Great West Road (CN925.1), Brentford, Middlesex TW8 9GS (GB).
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- Published:  
— with international search report
- (88) Date of publication of the international search report:  
16 October 2003
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **MEDICAMENTS WHICH ARE MODULATORS OF HM74 AND/OR HM74A ACTIVITY**

**Nucleic acid sequence for rabbit HM74A**

MNQHRPQSHFLEIDKKNCCVFRDDFIANVLPVGLGFVFGLLGNGLALWIFCFHLKSWKSSR  
IFLFNLAVADFLLIICLPFLTDNYMRKWWDWRFGDIPCRMFLMLAMNRQGSIIFLTVAVDYR  
FRVHPPHHAHNKISNRTAAAI SCLLWGV TIGLTVHLLRKRMLTQNGPANLCCSSFSICNTFRWH  
DAMFLLFEFFLPLAILFCSVRI VWSLRQRQMDRHVKIKRAINFIMVAVVFIIICFLPSVAVRMRI  
FWLLRTAGTQDCDVYRSVDLAFFITLSFTYMNSMLDPLVYVFSSPSFPNFFSALINRCLRRSPA  
GEPENNRSTSVELTGDPSTARSAPDALVAEPNGPRSPSYLVPNPR

(57) Abstract: A method of treating HM74A and/or HM74 mediated disease in an individual which comprises administering to the individual a therapeutic amount of a modulator of HM74A and/or HM74 activity.

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## INTERNATIONAL SEARCH REPORT

Intern      Application No  
PCT/GB 02/01671

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7    G01N33/50    G01N33/74    G01N33/68    C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7    G01N    C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 56820 A (SMITHKLINE BEECHAM CORP.) 17 December 1998 (1998-12-17) cited in the application page 3, line 8 -page 5, line 30 page 10, line 7 - line 14 page 13, line 8 -page 14, line 31 examples 4,6,8 claims 1-12	1-24
Y	LORENZEN A. ET AL.: "Characterization of a G protein-coupled receptor for nicotinic acid." MOL. PHARMACOL., vol. 59, no. 2, 2001, pages 349-357, XP002210646 the whole document --- -/--	1-24

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

30 May 2003

Date of mailing of the international search report

18/06/2003

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NOMURA H. ET AL.: "Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors." INT. IMMUN., vol. 5, no. 10, October 1993 (1993-10), pages 1239-1249, XP002911511 cited in the application abstract page 1247, column 2 ---	1-24
Y	ZINGONI A. ET AL.: "Isolation and chromosomal localization of GPR31, a human gene encoding a putative G protein-coupled receptor." GENOMICS, vol. 42, 1997, pages 519-523, XP002174048 abstract; figure 1 page 522, column 1 ---	1-24
Y	US 6 031 090 A (SMITHKLINE BEECHAM CORP.) 29 February 2000 (2000-02-29) the whole document ---	1-24
P,X	WO 01 61360 A (GLAXO GROUP LTD.) 23 August 2001 (2001-08-23) the whole document ---	1-24
P,X	WO 01 94385 A (BAYER AG) 13 December 2001 (2001-12-13) the whole document ---	1-24
T	WISE A. ET AL.: "Molecular identification of high and low affinity receptors for nicotinic acid." J. BIOL. CHEM., vol. 278, no. 11, 14 March 2003 (2003-03-14), pages 9869-9874, XP009011556 the whole document ---	
T	TUNARU S. ET AL.: "PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect." NATURE MED., vol. 9, no. 3, March 2003 (2003-03), pages 352-355, XP002242865 the whole document ---	

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	LEE D.K. ET AL.: "Discovery and mapping of ten novel G protein-coupled receptor genes." GENE, vol. 275, September 2001 (2001-09), pages 83-91, XP004307114 the whole document ---	
A	MARCHESE A. ET AL.: "Novel GPCRs and their endogenous ligands: expanding the boundaries of physiology and pharmacology." TIPS, vol. 20, September 1999 (1999-09), pages 370-375, XP004178194 the whole document ---	
A	STADEL J.M. ET AL.: "Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery." TIPS, vol. 18, November 1997 (1997-11), pages 430-437, XP004099345 the whole document ---	
A	WILSON S. ET AL.: "Orphan G-protein-coupled receptors: the next generation of drug targets?" BRIT. J. PHARMACOL., vol. 125, December 1998 (1998-12), pages 1387-1392, XP001010584 the whole document -----	

# INTERNATIONAL SEARCH REPORT

Int. application No.  
PCT/GB 02/01671

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.: 17-20  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.1

Although claim 22, due to the step of "administering to the individual a therapeutic amount of a modulator of HM74A and/or HM74 activity" is directed to a method of treatment of the human/animal body by therapy, the search has been carried out and based on the alleged effects of the compound.

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## Continuation of Box I.1

Claims Nos.: 22

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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## Continuation of Box I.2

Claims Nos.: 17-20

Present claims 17-20 relate to an extremely large number of possible activators/inhibitors/modulator. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds disclosed in the Examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/GB 02/01671

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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WO 0161360	A	23-08-2001	AU 3389501 A WO 0161360 A2	27-08-2001 23-08-2001
WO 0194385	A	13-12-2001	AU 6604901 A WO 0194385 A2 EP 1292677 A2	17-12-2001 13-12-2001 19-03-2003